# UPDATES ON THE NEUROPATHOLOGY OF SUDDEN UNEXPLAINED PERINATAL DEATH AND OTHER NEURODEVELOPMENTAL DISORDERS

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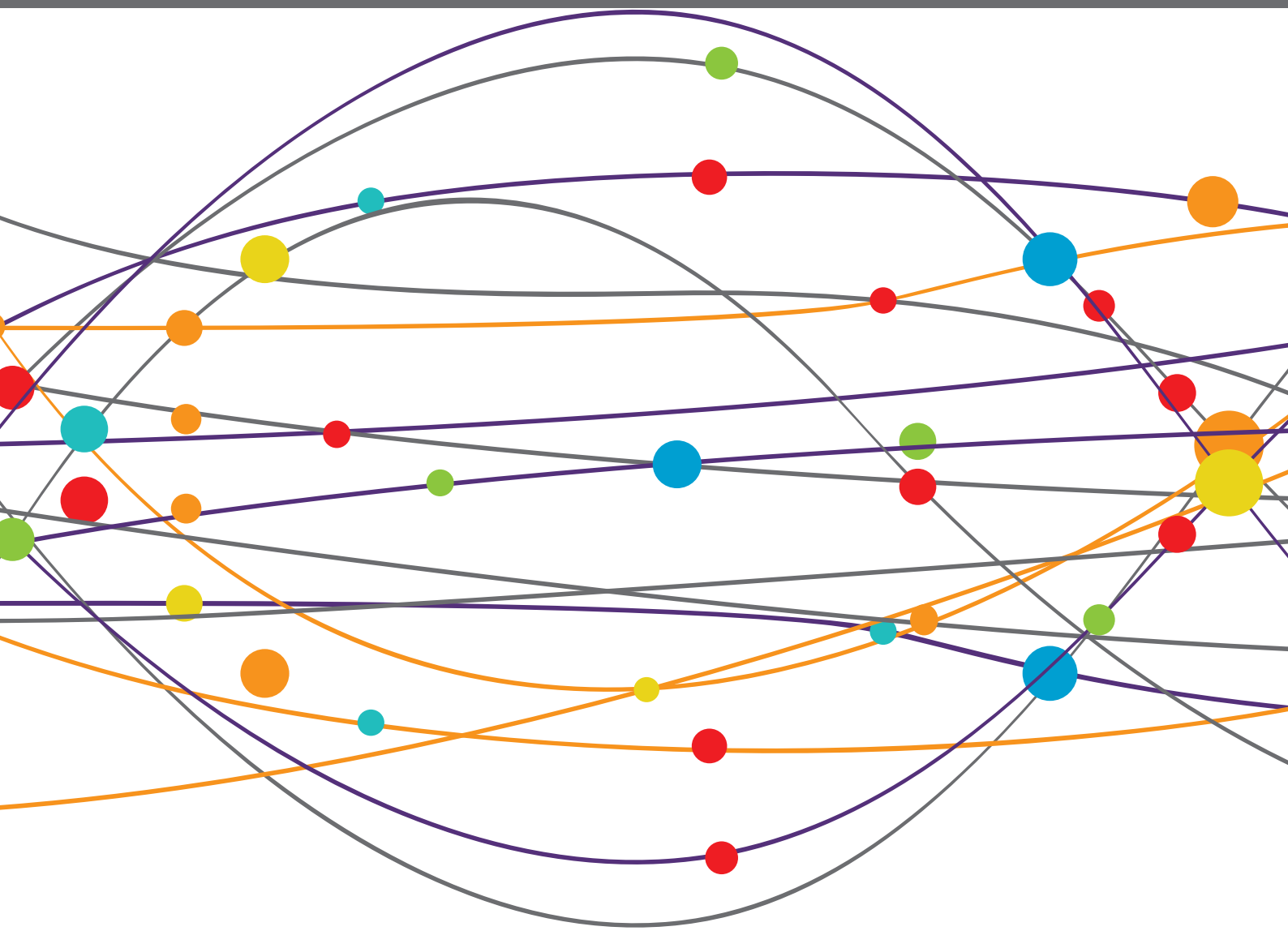

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# UPDATES ON THE NEUROPATHOLOGY OF SUDDEN UNEXPLAINED PERINATAL DEATH AND OTHER NEURODEVELOPMENTAL DISORDERS

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EDITORIAL
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# Editorial: Updates on the Neuropathology of Sudden Unexplained Perinatal Death and Other Neurodevelopmental Disorders

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**Keywords: neuropathology, sudden perinatal death, brain dysfunctions, neurodevelopmental disorders, environmental risk factors**

**Editorial on the Research Topic**

**Updates on the Neuropathology of Sudden Unexplained Perinatal Death and Other Neurodevelopmental Disorders**

Perinatal mortality includes both fetal demises (stillbirths) and deaths in the first week of life. Worldwide, there are over 6.3 million perinatal deaths a year, almost all of which occur in developing countries (1). Stillbirths and neonatal deaths have many common determinants, such as maternal diseases, adverse prenatal exposure, inadequate care or complications during pregnancy and delivery, and genetic mutations. The first few hours of postnatal life are also particularly sensitive, as this is a critical time for a successful transition from intrauterine to extrauterine life wherein newborns are less responsive and more vulnerable to stressors. In case of sudden perinatal death, an important first step is the post-mortem examination since it can reveal the pathology underlying the possible causes of this inauspicious event (2). However, no unique etiology can be determined in most pre- and post-natal deaths, even after accurate autopsy investigations. Detailed examination of the autonomic nervous system can often reveal subtle developmental alterations, potentially providing a plausible explanation for sudden death (3, 4). Other neurodevelopmental disorders characterized by profound dysautonomia caused by single gene mutations may also lead to sudden unexplained death in perinatal life and infancy. Some of these syndromes are also thought to involve a state of "immaturity" of autonomic control systems. They include Rett syndrome, Cyclin-dependent kinase-like 5 (CDKL5) deficiency disorder, Pitt-Hopkins syndrome, Congenital central hypoventilation syndrome (CCHS), and GRIN1-Related Neurodevelopmental Disorder (5–9).

The primary rationale for this Research Topic has been to advance the state of knowledge and expertise for investigating the neuropathology of unexplained perinatal deaths and, in particular:

- contribute to the identification of the pathogenic mechanisms underlying these deaths (especially if known gene mutations are absent), in the context of interactions with environmental risk factors (e.g., early exposure to smoking, air and water pollution, pesticides, food contamination) and neuropathological findings.
- facilitate the development of evidence-based prevention and management strategies to decrease the incidence of these inexplicable and devastating deaths.

Another aim of this proposal was to deepen knowledge regarding developmental brain dysfunctions that can manifest later in life as neuropsychiatric symptoms, impaired motor function,

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learning, language, or non-verbal communication behaviors such as crying, or autism spectrum disorders (10–12). A related goal was to inform literature on the role of early life experiences, often associated with pre- or peri-natal environment exposures, in shaping the developing brain and vulnerability for the later neurodevelopmental outcome.

This Research Topic presents a series of articles and reviews written by leading authors and covering most aspects of state-of-the-art research on the neuropathology of fetal and infant sudden death and other neurodevelopmental disorders.

The papers are grouped into two main sections, according to the purposes of the Research Topic:

1) Neuropathology of Sudden Unexplained Perinatal Death
2) Neuropathology of Other Neurodevelopmental Disorders

## 1) Neuropathology of Sudden Unexplained Perinatal Death

The articles are distributed in the following sub-sections:

**(a) Neuropathological research on sudden infant death**

- Blackburn et al. present an epidemiological study on the neuropathology of sudden infant death syndrome (SIDS). These authors point out that the underlying death mechanism reported in many studies is highly controversial. Using machine learning tools, they emphasize the existence of three distinct groups of SIDS, each with a unique peak age of death, and specific epidemiological and extrinsic risk factors.
- McGuone et al. provide a review on Sudden Unexplained Death in Childhood (SUDC), i.e., the unexpected death of a child over 12 months of age that remains unexplained after a thorough case investigation. The authors emphasize the scarce and unsatisfactory nature of literature on this subject, especially on the neuropathology of SUDC, and advocate greater interdisciplinary participation in research efforts to elucidate the underlying mechanisms, especially to institute preventive strategies.

**(b) Neuropathology of neonatal breathing**

- A thorough description of the multiple systems and mechanisms that underlie regulation of breathing and cardiovascular processes in newborns is represented by the contribution of Harper and Kesavan. This interesting article reviews, among other topics, the rationale and empirical evidence for use of peripheral locomotor muscle pacing as an alternative intervention for disordered breathing, an often overlooked mechanism to stabilize breathing.

**(c) Genetics**

- Congenital central hypoventilation syndrome (CCHS) is a genetic neurodevelopmental disorder with an autosomal dominant transmission caused by heterozygous mutations in the *PHOX2B* gene (8). Bachetti et al. consider its similarity to an idiopathic apparent life-threatening event (IALTE) and sudden unexpected infant death (SUID/SIDS). The authors report, for the first time, on the genetic screening of the three exons of the PHOX2B gene in Italian IALTE and SUID/SIDS cases. The study finds a statistically significant association between common 3′UTR variants in the exon 3 of the PHOX2B gene with these two pathologies, suggesting that CCHS, ALTE, and SUID/SIDS might be members of the same group of respiratory autonomic disorders of infancy.
- Ke and Chen report a rare case of a heterozygous nonsense mutation in the *CTNNB1* gene in a 15-month-old girl with a complex phenotype (dysmorphic features, microcephaly, hypotonia, development delay, retinal detachment, and polydactyly) associated with a neurodevelopmental disorder with spastic diplegia and visual defects.

**(d) Immunohistochemical studies**

- Alwazzan, Mehboob, Hassan et al. analyze the immunoexpression of Neurokinin-1 receptor (NK-1R), a receptor of tachykinin peptide substance P (SP), in miscarriage occurring in the first trimester of pregnancy. The authors highlight the involvement of SP/NK-1 receptor system dysregulations in these deaths and suggest the use of NK-1R antagonists to diagnose and treat spontaneous abortion.
- Another immunohistochemical study by Alwazzan, Mehboob, Gilani et al. shows that the alpha 7-nicotinic acetylcholine receptors (α7-nAChR) are highly expressed in the placenta and products of conception during the first trimester. These receptors could then be involved in sudden fetal deaths and complications of pregnancy.

**(e) Perinatal environment exposure**

- The harmful effects of maternal tobacco smoke on the nervous system of fetuses and newborns are covered in the mini-review by Bednarczuk et al. The authors examine literature in this field, emphasizing the mechanisms by which smoking in pregnancy can cause brain abnormalities in sudden intrauterine unexplained death syndrome (SIUDS) and SIDS and the need to educate women on these harms.
- Lucchini et al. investigate the effects of chronic tobacco and alcohol consumption during pregnancy on autonomic function in a wide population of fetuses at $\geq$34 weeks gestational age, by quantifying heart rate, heart rate variability, movement, and heart rate-movement coupling. The results of this study contribute to identifying new biomarkers and understanding the mechanisms underlying risk for adverse outcomes.
- The serious consequences of cigarette smoke and alcohol absorption in pregnancy are also the focus of the contribution by Vivekanandarajah et al. Through a multicenter longitudinal study and by using autoradiographic methods to highlight the nicotinic receptor binding in the brainstems of infants dying of SIDS, the authors provide evidence that developmental factors paired with changes in nicotinic receptor binding are related to the cause of death as well as exposure to maternal cigarette smoking.

**2) Neuropathology of Other Neurodevelopmental Disorders**

- Rett syndrome is caused by the loss of function of the transcription factor methyl CpG-binding protein 2 (MeCP2) (13). In an experimental study, Ward et al. compare hypoxic ventilatory responses in mice with cell-type specific knockout or rescue of *Mecp2*. The study suggests that MeCP2 expression in excitatory, inhibitory, or dopaminergic/noradrenergic neural cells is essential for normal hypoxic ventilatory responses. The authors explore the implications of these findings for the pharmacological treatment of breathing abnormalities and understanding the pathophysiology of sudden death in Rett syndrome.
- Another interesting contribution on the Rett Syndrome is given by the systematic review article of Singh et al. This study, which focuses on the autonomic features of sudden unexpected death in pediatric epilepsy, offers interesting indications on the management of epilepsy in patients with Rett Syndrome.
- Congenital central hypoventilation syndrome (CCHS) is the focus of the review article by Di Lascio et al. The study analyses various *in vivo* and *in vitro* approaches aimed at better understanding the CCHS molecular pathogenetic mechanism, in order to reduce the damage caused by the aberrant function of mutant PHOX2B and provide new therapeutic strategies.
- New insights into hypoxic ischemic encephalopathy (HIE) of the full-term newborn derive from the experimental study by Gotchac et al. The authors develop a new rodent model, using acute hypoxic cardiac arrest to induce HIE-like injury in post-natal rats. The study, which combines radiological, histological, and behavioral testing, finds that neurological changes that persist into adulthood, suggesting that the model may recapitulate milder forms of HIE. This model may be useful for understanding the long-term neurological and psychiatric consequences of oxygen deprivation during perinatal life.

Together, these publications expand current knowledge on the neuropathology of sudden unexplained perinatal/infant death and other neurodevelopmental disorders, such as the Rett syndrome and CCHS, thus allowing the broadening of the diagnostic criteria and preventive strategies. As editors of this Research Topic, we express our sincere gratitude to the authors who accepted the invitation to participate and for their significant efforts in identifying interesting approaches that explain the pathogenesis of these conditions. We would also like to thank the reviewers for their significant comments, elevating the quality of the submitted articles. Finally, we thank Frontiers and, in particular, the editorial office for essential support.

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REVIEW
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# Sudden Unexplained Death in Childhood: A Neuropathology Review

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Sudden Unexplained Death in Childhood (SUDC) is the unexpected death of a child over age 12 months that remains unexplained after a thorough case investigation, including review of the child's medical history, circumstances of death, a complete autopsy and ancillary testing (1). First defined in 2005, SUDC cases are more often male, with death occurring during a sleep period, being found prone, peak winter incidence, associated with febrile seizure history in ∼28% of cases and mild pathologic changes insufficient to explain the death (1, 2). There has been little progress in understanding the causes of SUDC and no progress in prevention. Despite reductions in sudden unexpected infant death (SUID) and other causes of mortality in childhood, the rate of SUDC has increased during the past two decades (3–5). In Ireland, SUID deaths were cut in half from 1994 to 2008 while SUDC deaths more than doubled (4). Surveillance issues, including lack of standardized certification practices, affect our understanding of the true magnitude of unexplained child deaths. Mechanisms underlying SUDC, like SUID, remain largely speculative. Limited and inconsistent evidence implicates abnormalities in brainstem autonomic and serotonergic nuclei, critical for arousal, cardiorespiratory control, and reflex responses to life-threatening hypoxia or hypercarbia in sleep (6). Abnormalities in medullary serotonergic neurons and receptors, as well as cardiorespiratory brainstem nuclei occur in some SUID cases, but have never been studied in SUDC. Retrospective, small SUDC studies with non-standardized methodologies most often demonstrate minor hippocampal abnormalities, as well as focal cortical dysplasia and dysgenesis of the brainstem and cerebellum. The significance of these findings to SUDC pathogenesis remains unclear with some investigators and forensic pathologists labeling these findings as normal variants, or potential causes of SUDC. The development of preventive strategies will require a greater understanding of underlying mechanisms.

**Keywords: sudden death, SUDC, SIDS, neuropathology, hippocampus, sudden unexplained death in childhood**

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## INTRODUCTION

Sudden unexplained death in childhood, (SUDC) is the sudden and unexpected death of a child 12 months or older that remains unexplained after a thorough case investigation, including review of the child's medical history, circumstances of death, a complete autopsy and ancillary testing (1). It is the 5th leading category of death in children aged 1–4 years, and resulted in 243 deaths in 2017 (7).

The rate of SUDC is increasing, even as mortality from sudden unexpected infant death (SUID) continues to fall (3–5). In Ireland, SUDC deaths more than doubled from 1994 to 2008, a period when SUID rates declined by 50% (4).

The paucity of SUDC literature precludes a detailed protocol-driven systematic review of the topic: published reports consist predominantly of limited autopsy-based case series or epidemiologic observations from a limited number of registries and cohorts. A PubMed search (08/10/2020) for sudden infant death syndrome or SUID revealed 15,355 articles but only 31 results for SUDC. Further, surveillance issues, including lack of standardized certification practices, and non-specific coding (R96 or R99) limit our ability to accurately assess SUDC incidence. These cases challenge the under-resourced and non-standardized U.S. medicolegal death investigation system, leaving a devastating impact on affected families (8, 9). To date, there has been little progress in understanding the causes or preventing SUDC.

**TABLE 1 |** Phenotypic features of SUID, SUDC, and SUDEP (10).

| | SUID | SUDC | SUDEP |
|---|---|---|---|
| Male Sex | + | + | + |
| Age | <1yr | 1–5 years | Any age |
| Death during apparent sleep | ++ | ++ | ++ |
| Prone position | ++ | ++ | ++ |
| Seizure history | +/– | +(FS) | ++ |
| Preterm birth/low birth weight | + | +/– | Unknown |
| Bedsharing/unsafe sleep environment | + | Unknown | Unknown |
| Illness/fever <48 h before death | + | + | – |
| Smoke exposure | + | Unknown | Unknown |
| Autopsy | WNL | WNL | WNL |
| Hippocampal microscopic changes | + | + | + |
| Serotonergic abnormalities | + | Unknown | + |

*SUID, Sudden unexpected infant death; SUDC, Sudden unexplained death in childhood; SUDEP, Sudden unexpected death in epilepsy; WNL, (within normal limits; minor findings insufficient to cause death are common); FS, febrile seizures; Associations are positive (+), strongly positive (++), or negative (–).*

## CLINICAL AND GENETIC FEATURES OF SUDC

SUDC is a diagnosis of exclusion that refers to a heterogeneous group of underling conditions. Phenotypic overlay with (SUID) and sudden unexpected death in epilepsy (SUDEP), suggests a biologic continuum between these sudden death syndromes (**Table 1**). A risk profile for SUDC has emerged over the past decade; most reported cases affect white non-Hispanic boys who were the product of a full-term pregnancy, with a median age of 1.6 years, (range 1–3 years) (1, 8). Population-based data of all-cause mortality affecting children aged 1–4, identifies ill-defined or unknown causes of death (R99), twice as frequently in African American children, underscoring that some SUDC cohorts have suffered selection bias in case ascertainment (7). Deaths are usually unwitnessed, occur during apparent sleep, and most children are discovered in a prone position, often face down (11). A history of febrile seizures (FS) is reported in up to one third of SUDC cases, (vs. ∼2–4% of controls); the median age at death tends to be greater in children with a FS history compared to those without, (24 vs. 19 months) (12). A high prevalence of FS history across SUDC cohorts generally comports with a spectrum of neuropathologic hippocampal observations of unclear biologic significance (**Table 2**). Not all SUDC cases with FS history are associated with hippocampal changes suggesting other mechanisms are likely relevant in some instances (12, 16). FS history might represent an independent marker for SUDC, with the caveat that not all FS are clinically obvious, and FS are probably underreported (2, 17). The estimated FS prevalence of ∼1–2% in the general pediatric population, and a low overall SUDC annual incidence, suggests additional factors modulate risk of SUDC in susceptible children, although data are limited regarding specific genetic variants influencing risk. As non-motor seizures may be associated with life-threatening apnea in early childhood the possibility that some SUDC cases represent SUDEP in children with undiagnosed epilepsy cannot be excluded (10). Further, a witnessed FS history is more frequent among explained pediatric deaths than children in the general population, although terminal seizures triggered by an exogenous stressor such as infection might still be relevant in these cases (2). A history of minor illness or fever in the 48 h prior to death, prior infection, minor head trauma, and peak winter incidence have also been associated with SUDC (1, 3, 18). By definition, autopsy examination and ancillary studies are negative or reveal only minor pathologic changes insufficient to explain death. Thus, detection of confirmed pathogenic variants by whole exome sequencing, such as cardiac channelopathy-susceptibility genes encoding sodium, potassium, or intracellular calcium channels, represent autopsy cases that become explained by genetic findings and are thereby excluded from a SUDC category of death (8). Pathogenic cardiac variants have been reported in up to 25% of sudden deaths lacking gross anatomic findings at autopsy (19–21). Although the genetic factors influencing SUDC vulnerability remain largely unknown, similarities with SUID and SUDEP, suggest seizure or cardiac related mechanisms are relevant in many cases. Moreover, exome sequencing of SUDEP cases has identified an excess of variants in genes that regulate ion channels in cardiac and brain tissue (22, 23). Perturbations of normal brain development resulting from *de novo* somatic mutations during embryonic or early post-natal development are increasingly recognized in multiple neurodevelopmental disorders including migration defects, epileptic encephalopathies, and other neuropsychiatric conditions, although a causal role in SUDC remains to be demonstrated (24, 25).

## NEUROPATHOLOGIC FINDINGS IN SUDC

SUDC likely represents a phenotypic endpoint for a heterogeneous group of underlying disorders, the mechanisms of which remain poorly defined. The proportional contribution of central nervous system disorders to this shared phenotype

**TABLE 2 |** Relative frequency of hippocampal findings and associated changes in SUDC in published series.

| Year | References | Dataset | Age (months) Median/range | Hippocampal abnormalities | Ethnicity | Gender | FS history | Asymmetry/ malrotation | Microscopic DG alterations | FDGB | Irreg DG | Ectopic GCs | GC loss | Subicular abnorm | Hipp Nn loss/ gliosis | Other NP findings |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2007 | Kinney et al. (3) | SDSRP | 18/13–36 | 5/23 | 4C; 1C/A | 4M;1F | 2/5 | 5/5 | 4/5 | – | – | – | – | 3/5 | 0/5 | SVN, Co. NeoCx, MD |
| 2009 | Kinney et al. (13) | SDSRP | 20.4/12–70.8 | 16/26 | 13C; 2A; 1 other | 8M;8F | 7/16 | 5/26 | 10/26 | – | – | – | – | 4/26 | 2/26 | IOD (3/16); FLC (2/16); WM and PAG heterotopia, (2/16) |
| 2016 | Kinney et al. (14)* | SUDP at BCH; SDSRP | 23.7–44.9 | 17/17 | 17C | 10M;7F | 11/17 | – | 17/17 | 16/17 | 10/17 | 17/17 | 16/17 | 7/17 | 8/17 (HG) | FCD (9/16); Hamartia (4/17); heterotopia (8/17); IOD (3/16); OH (7/16); Arcuate MD (5/16); cerebellar MD (3/16); FP (3/16) |
| 2016 | Hefti et al. (16) | SDSRP; BCH | 12–79.1 | 42/79 | 64C | 43M;34F | 24/77 | 17/42 | 63/79 | 38/79 | 48/79 | 63/79 | 55/79 | – | 43/79 (HG) | WM heterotoia (5/77); IOD (1/49); encephalitis (1/77); HIC (22/77) |
| 2020 | McGuone et al. (15) | SUDCRRC | 33.3/12–142 | 19/20 | 16C; 3H/W; 1EA/H/W | 8M;12F | 12/20 | 0/20 | 19/20 | 3/20 | 6/20 | 3/20 | 6/20 | 0/20 | 0/20 | Incidental minor findings (2/20) |

*FS, personal febrile seizure history; DG, dentate gyrus; FDGB, focal dentate gyrus bilamination; GC, granule cell; Nn, neuronal; NP, neuropathologic; SDSRP, San Diego SUDC Research Project; SUDP, Sudden Unexplained Death in Pediatrics Program; BCH, Boston Children's Hospital; SUDCRRC, SUDC Registry & Research Collaborative; C, Caucasian; A, Asian; AA, African American; H, Hispanic; EA, East Asian; M, male; F, female; *age 1 to < 6 yrs; HG, hilar gliosis; SVN, subventricular neuroblasts; Co. NeoCx. columnar neocortex; MD, microdysgenesis; IOD, inferior olivary dysplasia; FLC, fetal like cortex; WM, white matter; PAG, periaqueductal gray; FCD, focal cortical dysplasia; OH, olivary heterotopia; FP, fused pyramids; HIC, hypoxic ischemic change.*

is unknown. A complete autopsy is the gold standard for understanding causes and consequences of lethal disease, and a detailed examination of the brain is necessary to identify potentially unexpected neurologic causes of death. This is also critical to power research to inform future preventative interventions. However, in the United States sudden unexpected pediatric deaths are investigated by a non-uniform medico-legal investigation system consisting of over 2,000 autonomous jurisdictions run by a mixture of physician medical examiners and lay coroners (26). At a diagnostic level an absence of procedural guidelines for pediatric death investigation beyond infancy, combined with uneven access to pediatric and neuropathology expertise has resulted in large variation of autopsy standards, with neuropathologic examinations that are frequently insufficient. Finally, although essential for public health surveillance, the medical death investigation system is under-resourced, short-staffed, and chronically under-funded, problems which have deepened during the opioid epidemic, and COVID-19 novel coronavirus disease pandemic. Together, these issues have conspired to severely limit progress in understanding SUDC pathogenesis.

One audit of SUDC autopsy practice found improved reporting when autopsies were performed by pediatric pathologists compared to non-specialists (27). Protocols for the investigation of unexpected infant death and recently published guidelines for SUDC are available; however, implementation remains problematic due to the persistent and systematic challenges described above (8, 28–31).

Neuropathologic findings in SUDC are particularly relevant when a seizure is postulated as the immediate mechanism of death. There are no specific autopsy findings to confirm an acute seizure, and, if a death is unwitnessed, as occurs in more than 90% of cases, the diagnosis becomes speculative without strong supporting circumstantial evidence (32). However, stigmata of convulsive seizures may be absent. General autopsy findings such as tongue biting and urinary incontinence are occasionally seen in older children and adults; however, these only occur in a minority of cases and lack specificity (2, 33–35). Further, young children can have non-convulsive seizures that cause respiratory arrest and near-death events (36, 37). Finally, even in adults with epilepsy, sudden death can occur during video electroencephalogram (EEG) monitoring without clinical or electrographic evidence of a seizure, and autopsies reveal no alternative causes.

Neuropathological research has emphasized a biologic continuum between SUDC and SUID, focusing on two inter-connected brain regions: the hippocampal formation, a limbic system hub connecting to brainstem in the central autonomic network, and brainstem cardiorespiratory centers (38–40). Brainstem serotonergic and autonomic nuclei are critical in controlling arousal as well as cardiorespiratory centers that respond to life-threatening hypoxia or hypercarbia during sleep. Although extensively studied in SUID, in older children research has instead focused mainly on the hippocampal formation (6, 41, 42). In SUDC, neuropathological studies have focused on hippocampal abnormalities, yet no study has examined the role of medullary serotonergic brainstem neurotransmission (14, 43).

Although epidemiologic data support a link between neuropathologic changes, FS history, and SUDC, the nature of this association is poorly understood. Early exploratory analyses of the San Diego SUDC Research Project, (SDSRP), a multicenter initiative created to characterize the main pathologic features and risk profile of SUDC, were key to elucidating the initial relationships between external and microscopic abnormalities of the hippocampus, sudden death during apparent sleep, and FS history (1, 3, 13). Subsequent analyses have expanded on this original hippocampal phenotype to identify the key elements of Hippocampal Malformation Associated with Sudden Death, (HMSASD) (16). The defining features of HMASD include external malrotation or asymmetry of the hippocampus, and a cluster of developmental lesions centered on the dentate gyrus (DG) (**Table 2**). Additional analyses have emphasized alterations of the granule cell layer (GCL) including granule cell dispersion (GCD) and focal dentate gyrus bilamination (FDGB) as key findings (**Figure 1**) (14, 40). Similar GCL alterations occur in hippocampal sclerosis in temporal lobe epilepsy, where FDGB is associated with more severe disease (44). Whether these changes are necessary or sufficient to cause seizures in SUDC remains unproven and controversial (16, 45, 46). Unlike temporal lobe epilepsy, hippocampal sclerosis is rare or never occurs in SUDC while acquired hippocampal injury (e.g., neuronal loss or gliosis) is uncommon in SUDC (13, 15, 16).

The strong association between hippocampal alterations and FS history has prompted speculation that the mechanism of SUDC could be a terminal seizure-like event, reminiscent of sudden unexpected death in epilepsy (SUDEP) (2, 12). A key unanswered question, however, is the biologic relevance of GCL alterations as it remains unclear whether these changes are a cause or a consequence of seizures. Moreover, the extent to which GCL alterations overlap with normal anatomic variation requires further clarification. Evidence in support of a developmental basis of hippocampal changes is inferred primarily from imaging and autopsy data. Rare case reports of bilateral GCD in infancy show an association with cortical polymicrogyria in some cases, although EEG data and seizure history were lacking (47). Many autopsy reports are limited by insufficient correlative clinical data and subclinical seizures usually cannot be excluded, raising doubts as to the strength of the evidence in support of a developmental hypothesis. Additionally, inconsistent histologic classification schemes, absence of agreed upon definitions, and non-uniform sampling protocols make subjective interpretations of GCL alterations problematic without additional and detailed morphometric studies (15, 48). Some experimental data suggest seizure induced neurogenesis and altered neuronal migration as playing a causal role in GCL alterations (49–51). However, other experimental data suggest GCL alterations as causal in seizure genesis, so the question of cause vs. effect remains unresolved (52, 53).

A retrospective analysis of a large SUID cohort in an urban medical examiner setting, found FDGB in ∼40% of infants, and ∼8% of explained deaths (43). A similar distribution of GCL alterations has consistently been reported in explained "control" groups of SUDC cohorts (15, 16). The apparent high prevalence of GCL alterations in pediatric deaths with

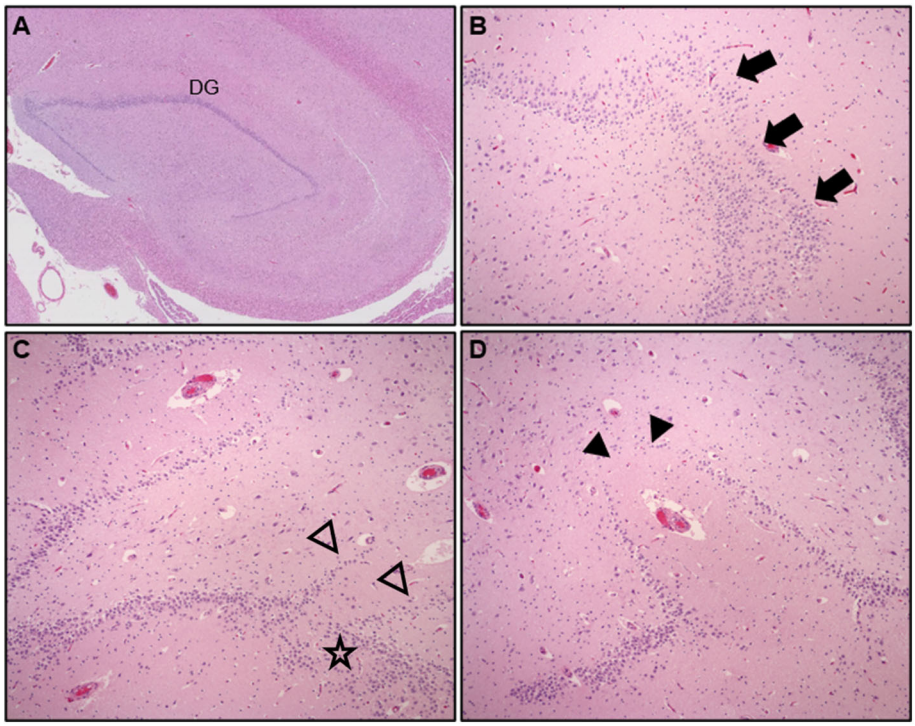


**FIGURE 1 |** Hematoxylin & Eosin (H&E) stained hippocampal tissue sections at lateral geniculate nucleus level showing **(A)** compact arrangement of the normal dentate gyrus (DG), low power, **(B)** granule cell dispersion (GCD), with focal dentate gyrus bilamination, arrows (mag = 200X), **(C,D)** adjacent hippocampal sections from a child with explained cause of death showing complex DG architecture, abnormal linear bands of granule cells (open arrowheads), GCD, (star), and focal DG granule cell loss, (arrowheads), (mag = 200X).

explained causes raises questions about the etiologic specificity, and therefore biologic relevance, of the role of GCL alterations in the SUDC brain. Observational and experimental protocols designed to evaluate GCL alterations are necessarily biased toward hippocampal sampling which might account for a trend toward increased sensitivity to over-interpret normal anatomic variants in some cases as the spectrum of normal variant anatomy during hippocampal development is poorly defined and prevalence data for the general pediatric population are lacking. A recent morphometric analysis of archived autopsy pediatric material found no correlation between GCD and seizure history (54). Thus, the significance of GCL alterations remains uncertain.

Autopsy data are an endpoint and have limited capacity to inform our understanding of the natural history of a disease process. However, the frequent association of GCL alterations and FS history in SUDC has prompted some researchers to suggest that GCL is a marker of seizure vulnerability in early life, potentially through age-dependent mechanisms involving altered limbic-brainstem connections (38). Multiple hippocampal structural abnormalities were reported in the most recent SDSRP cohort report (16). Half of SUDC cases showed HMASD with or without a FS history and the most frequent histologic findings in this group were GCD, FDGB, irregularity of the DG, and ectopic granule cells. The investigators suggested that a malformed hippocampus could predispose to seizure development during sleep when seizure risk is greatest, however, this analysis also revealed comparable SUDC rates in cases that lacked hippocampal changes, raising doubts whether this finding is signal or noise. Notably, almost half of the cohort did not have hippocampal alterations and 27% did not have febrile seizures. However, researchers have since raised concerns about the reliability of the association in the SDSRP study, as hippocampal tissue was unavailable for analysis in approximately half the cases (46). Other methodologic issues included an incomplete dataset populated by self-referred cases that lacked standardized death investigation, with frequent limited brain examination and sampling (46, 55).

Standardized, unrestricted whole brain examination is essential to elucidate the structural abnormalities, if any, in the SUDC brain. Thus, the SUDC Registry and Research Collaborative (SUDCRRC) at NYU Langone Health undertook a 5-year prospective analysis of 20 SUDC cases accrued through a registry where systematic sampling of whole brains was performed with blinded independent reviews conducted by neuropathologists (15). The observations were supplemented by 3T-MRI imaging, and whole exome sequencing to identify pathogenic variants relevant to death. Whole brain analysis revealed hippocampal alterations of the DG as the most frequent microscopic alteration across all examined brain regions, although these were not specific to SUDC as three cases with these findings died from pathogenic genetic cardiac variants. The most frequent DG alterations included alternating

thickness, irregular configuration, focal GCL loss, and ectopic neuronal clusters. Unlike prior reports, GCD or FDGB, were not prominent findings, raising questions about the importance of this finding as a morphologic marker of SUDC in this cohort. It was not immediately apparent why GCL alterations were not prominent in this series as 30 individual hippocampal observations (15 on either side) were scored based on the defining features of HMASD (14). As morphometric analysis was not conducted the possibility of heightened sensitivity and observer bias to specific lesions, particularly in more subtle cases, cannot not be entirely excluded. Alternatively, and perhaps more likely, is that prior studies suffered observer bias as there was greater tendency to consensus-based decision making without blinding.

A history of FS was present in ∼60% of these SUDC cases, substantially more than previously reported cohorts and likely reflecting referral bias from forensic institutions with limited access to neuropathology expertise. The registry provided *ex vivo* MRI imaging and brain examination by a board-certified neuropathologist. A history of subclinical seizures could not be excluded in one patient with FDGB who had no FS history. Importantly, this study showed no consistent distribution of microscopic findings outside the hippocampus, such as cerebellar cortical dysplasia and anomalous inferior olivary nuclei, findings which were occasionally seen in other cohorts. Although a well-conducted prospective cohort study, this study still suffered limitations. The small sample size due to the rarity of SUDC, limited access to true normal controls such as pediatric trauma, or children without medical comorbidities, and a hippocampal sampling bias, necessarily require that conclusions about the relative contribution of hippocampal abnormalities in SUDC should still remain tentative.

Neuropathologic research in SUDC has focused primarily on the hippocampus, with speculative mechanisms of a limbic-brainstem network disorder contributing to epileptogenesis and brainstem dysfunction in susceptible children (48, 56). These hypotheses remain untested and the brainstem is conspicuously understudied in SUDC. Multiple neurotransmitter defects of brainstem respiratory and autonomic pathways were identified in SUID brains, with abnormalities of the medullary 5-Hydroxytryptamine (serotonin) (5-HT) system implicated as a major network vulnerability for sudden death in infancy (6). Notably, the same group that has championed the role of hippocampal abnormalities in SUDC and SUID had previously highlighted the role of medullary serotonin and other brainstem abnormalities, including structural abnormalities in the inferior olive, reactive gliosis in the medulla, interleukin 6 and nicotinic and muscarinic receptor abnormalities in the medulla (57–59).

Medullary 5-HT neurons are central respiratory chemosensors and contribute to arousal and key autonomic functions (41, 60). Further, altered serotonergic transmission is altered in animal models of hippocampal dysfunction and epilepsy, and serotonin efferents from brainstem raphe neurons to the DG regulate GCL neurogenesis and cell migration in early hippocampal development (43, 61). It remains unclear whether hippocampal structural changes in SUDC can result from disturbed neurotransmission due to an intrinsic brainstem serotonergic defect arising during early development. Further research is necessary to clarify the significance of limbic-brainstem connections in SUDC, and whether GCL alterations could represent a potential marker of underlying 5-HT brainstem defects. The challenge is that studies typically focus on one or two anatomical structures or functional assays, and the brain has hundreds of networked structures and scores of neurotransmitters, neuromodulators and receptors. Viewing a tiny fraction of a complex picture may lead to spurious conclusions perhaps most dangerous, they may close the door to more relevant pathogenic structures or functional systems.

## POTENTIAL NEUROLOGIC MECHANISMS OF SUDC

The functional basis of SUDC remains poorly defined partly because witnessed deaths are extremely rare, but also because relevant animal models are lacking. SUDC is an endpoint for diverse disorders, some of which may be seizure driven and display phenotypic overlay with SUDEP. To date, the only one witnessed SUDC case report was a 20-month-old toddler undergoing epilepsy monitoring in whom febrile status epilepticus was followed by bradycardia (2). One proposed mechanism of SUDC associated with FS includes thermal sensitivity of the developing brain central homeostatic network (40). The potential for seizure-like events precipitated by exogenous stressors remains speculative and shares similarities with the triple risk model of SUID (5). Although the range of potential stressors is unknown, epidemiologic data have identified that SUDC occurs more frequently in winter, and ∼75% have a history of a recent minor illness within 2 weeks before death (1, 11). Moreover, minor inflammatory infiltrates are common in the lungs and other organs at autopsy, suggesting that post-infectious immunologically mediated processes could also be relevant, although these findings are not specific and occur commonly in other children with well-explained causes of death (1). A history of minor blunt head injury identified in one quarter of SUDC cases from the initial SDSRP cohort, suggested a potential role for post-concussive mechanisms, although trauma has not been reported as a correlative risk for SUDC in subsequent analyses (1). Retrospective questions of parents who have lost a young child may also be biased by a desire to "find the answer," potentially biasing recall of minor head injuries as more serious. In addition, shared circumstantial features of early childhood deaths, including a tendency for death to occur in a prone position during a period of apparent sleep suggests pathophysiologic mechanisms related to sleep and development might be important, at least in some instances.

SUDC and pediatric SUDEP share several features (14, 40, 62). SUDEP is a diagnosis of exclusion that refers to the sudden, unexpected death of a person with epilepsy in whom an autopsy does not reveal a structural or toxicologic reason for death (63). Seizure-induced autonomic or respiratory disturbances are implicated in most SUDEP deaths (10, 48). The age of epilepsy onset influences SUDEP risk, which increases with earlier onset epilepsy, implying that developmental mechanisms

might influence vulnerability (62, 64). Hippocampal anomalies of the DG, analogous to SUDC, occur in some SUDEP brains, but are not common in SUDEP and many epilepsy patients who die from other causes have hippocampal abnormalities (32). These changes also occur in experimental animal models where hippocampal stimulation evokes cardiorespiratory effects (48, 65–67). In some cases, a distinction between SUDC and SUDEP may be semantic as some SUDC cases had epilepsy syndromes retrospectively diagnosed based on genetic and other data, but epilepsy was not recognized before death.

The mechanisms of death in SUDEP are poorly understood because few observed cases have occurred during epilepsy monitoring. Most SUDEP cases occur during apparent nocturnal sleep and multifactorial mechanisms likely include seizure-induced autonomic disturbance, impaired arousal, apnea, cardiorespiratory depression, and brainstem dysfunction (10). The role of these heterogeneous mechanisms in SUDC pathogenesis remains poorly defined. The Mortality in Epilepsy Monitoring Units Study, (MORTEMUS), identified 11 SUDEP patients who died after terminal generalized tonic-clonic seizures during epilepsy monitoring (68). Cardiorespiratory data were available for 9 patients who displayed a consistent pattern of seizure associated cardiorespiratory collapse that included postictal tachypnea and cardiac dysfunction followed by central apnea, severe bradycardia, and asystole. Imaging and volumetric assessments of SUDEP brains have demonstrated volume changes affecting brain regions responsible for modulating cardiorespiratory control, including atrophy in areas that protect against cardiorespiratory collapse, and increased volume in areas that predispose to apnea and/or hypotension (69, 70). Comparable volumetric analyses in SUDC cohorts are lacking. As with SUID, abnormalities of the serotonergic system and other neurotransmitter systems are implicated in SUDEP; the 5-HT type 2c receptor knockout mouse is an animal model of SUDEP with spontaneous seizures leading to respiratory arrest and death within seconds of onset (10, 71). Medullary serotonergic neurons function as chemosensors responsible for stimulating breathing and arousal in response to hypercapnia and may contribute to ictal apnea and respiratory arrest if inhibited by seizures (72, 73).

Although the role of serotonergic network dysfunction in SUDC pathogenesis remains unknown, the requirement for hippocampal transmission from brainstem serotonergic neurons for early DG neurogenesis suggests hippocampal alterations may be a morphologic marker of altered brainstem serotonergic function (43, 61). Given these converging strands of evidence, hippocampal abnormalities have emerged as a potential risk factor for seizure-like mechanisms responsible for SUDC, potentially mediated through apneic seizures and cardiorespiratory dysregulation (40). Some researchers suggest these changes might be considered as "pre-epilepsy" because of a presumptive increased risk for seizure progression, a lesion termed "epilepsy *in situ*" (65). However, there is limited evidence to directly support the role of hippocampal dysfunction in SUDC pathogenesis. The structural changes in the hippocampus may be secondary to interictal or ictal discharges (44).

## EPIDEMIOLOGIC FEATURES OF SUDC

Understanding risk factors and the underlying biologic mechanisms of SUDC is essential for informing effective public health surveillance and developing strategies to mitigate risk in susceptible children. Successful educational and public health campaigns have substantially reduced the burden of SUID (11, 74). In contrast, SUDC has received relatively little attention, partly because it is such a rare condition The Sudden Death in the Young registry, under the auspices of the Centers for Disease Control and Prevention and the National Institutes for Health is responsible for tracking unexplained infant as well as childhood deaths, although just 13 states or jurisdictions actively participate in this program (75, 76). Further, the low incidence of SUDC creates additional challenges for healthcare providers who often rely on an interdisciplinary approach to recently bereaved families, and interface with the medico-legal system (8, 11).

Before 2005, few studies considered unexplained post-infancy death other than brief descriptions in occasional medico-legal series primarily concerned with distinguishing natural from unnatural death (77–80). Brain findings were generally not discussed unless gross pathologic abnormalities were seen; i.e., hippocampal histology was not reported. However, many of these cases would likely be re-classified in the modern era through contemporaneous genomic analyses. Molecular testing has yielded high rates of pathogenic cardiac variants in up to 25% of cases of published sudden death cohorts, and ∼12% of explained sudden pediatric deaths which possibly explain cause and mechanism of death in some cases, underscoring the necessity of comprehensive ancillary testing to identify causes (15, 20, 21). Moreover, non-neurologic or cardiac mechanisms may also account for a subset of SUDC deaths.

A large population study of ∼10,000 children enrolled in a SUID study identified 5 unexplained deaths in children 1–5 years of age (81). Two deaths were associated with apnea and convulsions. Another retrospective analysis of childhood mortality by pathologists in England found 5 unexplained deaths among 1,012 sudden pediatric deaths, although brain findings were not reported for unexplained cases (82). Other studies have identified similar trends, and countries that routinely track SUDC mortality report a gradual increase in incidence over time (4, 83–85).

## CONCLUSIONS

SUDC is a major cause of unexpected mortality in toddlers that devastates families. Clinicopathologic correlation remains elusive as SUDC lacks a distinct pathoanatomic signature—in the brain or elsewhere. Most SUDC fatalities are unwitnessed and little is known of the immediate pathophysiologic disturbances preceding death, although seizure-like mechanisms analogous to SUDEP may explain some cases with a FS history (12). Accrual of SUDC cases through SUDC registries has enabled researchers to construct a predicted profile of a typical SUDC child, although this does not capture the heterogeneity of underlying disorders that are likely responsible for sudden death in children. Retrospective studies with non-standardized

methodologies have demonstrated a high rate of hippocampal abnormalities associated with FS history, and SUDC. These studies have been limited by sample size and lack of appropriate controls, which limit conclusions that can be drawn. Future detailed studies are necessary to elucidate the mechanism of hippocampal alterations, and explore connections with brainstem dysfunction. A developmental continuum has been proposed as a framework for SUID and SUDC, with the expectation that future advances in imaging and genomics will help resolve convergent mechanisms and pathways of relevance to both groups (39). Ultimately additional and adequate support for a resource-limited medicolegal death investigation system is necessary to allow the extensive ancillary testing required to fully investigate and identify and exclude underlying pathologies in putative SUDC deaths. Greater interdisciplinary participation in research efforts is also crucial to elucidate underlying mechanisms, identify children are at increased for sudden death, and to institute appropriate screening and preventative strategies.

## AUTHOR CONTRIBUTIONS

All authors were involved in the conception, literature review, writing, and editing.

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# The Role of Maternal Smoking in Sudden Fetal and Infant Death Pathogenesis

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Maternal smoking is a risk factor for both sudden infant death syndrome (SIDS) and sudden intrauterine unexplained death syndrome (SIUDS). Both SIDS and SIUDS are more frequently observed in infants of smoking mothers. The global prevalence of smoking during pregnancy is 1.7% and up to 8.1% of women in Europe smoke during pregnancy and worldwide 250 million women smoke during pregnancy. Infants born to mothers who smoke have an abnormal response to hypoxia and hypercarbia and they also have reduced arousal responses. The harmful effects of tobacco smoke are mainly mediated by release of carbon monoxide and nicotine. Nicotine can enter the fetal circulation and affect multiple developing organs including the lungs, adrenal glands and the brain. Abnormalities in brainstem nuclei crucial to respiratory control, the cerebral cortex and the autonomic nervous system have been demonstrated. In addition, hypodevelopment of the intermediolateral nucleus in the spinal cord has been reported. It initiates episodic respiratory movements that facilitate lung development. Furthermore, abnormal maturation and transmitter levels in the carotid bodies have been described which would make infants more vulnerable to hypoxic challenges. Unfortunately, smoking cessation programs do not appear to have significantly reduced the number of pregnant women who smoke.

**Keywords: sudden infant death syndrome - SIDS, sudden intrauterine unexplained death syndrome, hypoxia, hypercarbia, brainstem, carotid bodies**

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## INTRODUCTION

Sudden intrauterine unexplained death syndrome (SIUDS) is defined as the "sudden death of a fetus after the twenty-fifth week of gestation and sudden infant death syndrome (SIDS) the sudden death of an infant under the age of 1 year, which is unexplained following thorough examination of the clinical case, history, death scene and autopsy (1). Although the rates of SIDS have decreased during recent years due to successful public health campaigns (2, 3), the rates of SIUDS have remained largely unchanged and account for > 50% of stillbirths (1, 4–6). Despite modern advances, the pathophysiology remains unexplained, but is likely to be multi-factorial (7). The Triple Risk Model has been proposed as a potential explanation of SIDS; that is a vulnerable infant within a critical developmental period is exposed to an exogenous stressor (8). There have been many suggestions for the mechanism resulting in mortality including abnormal cardiorespiratory control, autonomic

nervous system abnormalities and failure of arousal from sleep (7, 9, 10). Unfortunately, much less is understood about the pathogenesis underlying SIUDS, although there is likely to be an overlap (1).

Maternal smoking is a modifiable risk factor as both SIDS and SIUDS are more frequently observed in infants of smoking mothers (7, 11). A nationwide survey in New Zealand demonstrated smoking in pregnancy and/or bed sharing were the most important risk factors for sudden unexplained infant death regardless of ethnicity (12). A dose-dependent relationship exists, with an increasing risk of sudden death with increasing daily maternal cigarette consumption (13, 14). Between one and 20 cigarettes, the probability of sudden unexplained infant death increased linearly in one study. Furthermore, the risk was reduced in mothers who quit or reduced their smoking in pregnancy (14). If causality is assumed, 22% of sudden unexplained infant deaths in the United States of America can be attributed directly to maternal smoking in pregnancy (14). Worryingly, then the global prevalence of smoking during pregnancy is 1.7% with up to 8.1% of women smoking during pregnancy in Europe and 250 million women smoking during pregnancy worldwide (15, 16). Importantly, smoking cessation programs appear not to have significantly reduced the number of pregnant women who smoke (16–18). In a large randomised controlled trial involving 1,050 participants, nicotine patches did not increase the rate of abstinence from smoking until delivery or the risk of adverse pregnancy or birth outcomes, but compliance rates were low being 7.2% for nicotine patches and 2.8% for placebo patches used for more than 1 month (19).

The aim of this review was to highlight the role of maternal smoking in the pathogenesis of sudden fetal or infant death. We have, therefore, examined the literature to identify the mechanisms by which smoking in pregnancy could cause harm and the functional and structural abnormalities.

## PHYSIOLOGICAL ABNORMALITIES IN INFANTS OF MOTHERS WHO SMOKED

Newborns exhibit a biphasic response to hypoxia, with an increase in ventilation initially, followed by a later decrease in respiratory rate (20). In infants of mothers who smoked during pregnancy, a steeper ventilatory deceleration and a shorter time to reach the lowest oxygen saturations in response to hypoxia has been observed (20) and a greater decline in minute volume in response to a hypoxic challenge (21). These differences remained significant when adjusting for differences in birthweight, age and sex (20, 21). Furthermore, maternal smoking and substance abuse particularly impair the response to hypoxia in the prone position (22). This maladaptive response can be explained by the infant's chronic exposure to fetal hypoxia, leading to persistent, inappropriate activation of the respiratory neural network underlying the biphasic ventilatory response (21, 23). Furthermore, infants exposed to smoking *in utero* have been shown to display significant arousal abnormalities, taking longer times to wake when exposed to exogenous stressors, such as hypoxia, thus increasing vulnerability to SIDS (24, 25). Additionally, infants of smoking mothers also displayed a dampened ventilatory response to hypercapnia, which may be secondary to abnormalities within the central chemoreceptors, such as the locus coeruleus (26–28).

Abnormal cardiac function has been demonstrated in infants of smoking mothers. Increased vascular, cardiac and blood pressure reactivity in response to inhalation of 4% carbon dioxide or passive head tilt to 60 degrees has been demonstrated in infants of smoking mothers (29). The normal response to hypoxia is a tachycardia mediated by cardiac vagal motor neurons activated by lung stretch receptors (30). Infants of mothers who smoked during pregnancy showed a significantly lower increase in heart rate in comparison to infants of non-smoking mothers (20). Those findings could be explained by the enhanced inhibitory receptors on the smoke-exposed infant's heart and a dampened response to circulating catecholamines (31, 32). Nicotine exposure in pregnant rats led to a shift in expression of autonomic receptors in the offspring's heart, with a greater proportion of receptors with inhibitory effects on the heart (32). Consequently, the heart's response to circulating adrenaline was blunted by prenatal nicotine exposure (29, 32). In combination with an already reduced catecholamine production (31), the sympathetic response to stress following *in utero* smoke exposure was significantly dampened (33).

Perinatal exposure in mild 5-HT deficient rat neonates has been shown to exacerbate autoresuscitation failure (34).

## MECHANISM OF HARM CAUSED BY MATERNAL SMOKING

Tobacco smoke contains a variety of hazardous components, but its harmful effects are mainly mediated by the release of carbon monoxide and nicotine. Carbon monoxide can diffuse across the placental barrier and enter the fetal circulation. There it binds to fetal haemoglobin, causing an increase in carboxyhaemoglobin, resulting in a left shift of the oxygen dissociation curve and thereby reducing oxygen release into the fetal tissues (35). The resulting hypoxia in the fetus can be detrimental to development, especially for organs such as the lungs and brain (7, 36, 37). As a result of chronic hypoxia, aerobic cellular respiration is reduced leading to anaerobic metabolism and greater oxidative stress (38). This leads to higher levels of reactive oxygen species, which can directly damage and fragment DNA, ultimately resulting in apoptosis of developing cells (38, 39). Evidence of hypoxic injury in infants of smoking mothers is greater astrocyte gliosis (40, 41). Additionally, greater levels of iron accumulation in the central nervous system (CNS) have been shown; the iron may be the catabolic product of maternal methaemoglobin, a biomarker of oxidative stress (42). The combination of maternal anaemia and smoking appears to increase the risk of sudden perinatal death, likely by amplifying the hypoxic effect of smoking as maternal anaemia was not found to be an independent risk factor for sudden perinatal death (7, 43, 44).

Nicotine can enter the fetal circulation and affect multiple developing organs, including the lungs, adrenal glands and the brain (31, 45, 46). The damaging properties of nicotine are

further enhanced by its prolonged half-life as a consequence of the immature fetal liver (33, 45, 47). Nicotine mimics the effects of the neurotransmitter acetylcholine (ACh) and acts on endogenous nicotinic ACh receptors (nAChR). Importantly, nicotine can cross the blood-brain barrier due to its lipid solubility and there have extensive effects due to the widespread expression of nAChRs in the CNS (18, 48, 49). In a review it was highlighted that the major brainstem sites where the expression level of nAChRs are consistently affected include those that play vital roles in cardiorespiration (hypoglossal nucleus, dorsal motor nucleus of the vagus nucleus of the solitary tract), chemosensitisation (nucleus of the solitary tract, arcuate nucleus) and arousal (rostral mesopontine sites such as the locus coeruleus and nucleus pontis oralis). Nicotine affects α7-nAChRs which play a vital role in the development of the brainstem regions receiving cholinergic projections in perinatal life (50). It can also bind to nACHRs in peripheral organs, such as the lung (45). In developing organs, the effect of exogenous nicotine on nAChR disrupts the natural sequence of cholinergic signalling, which is crucial for normal development, and can result in inhibition of DNA synthesis, abnormalities in gene expression and ultimately cell apoptosis (46, 51). This results in early termination of cell development and abnormal functioning of surviving cells (52). In the CNS, nicotine exposure has been shown to lead to neuronal hypoplasia, as well as an inappropriate early terminal differentiation of developing neurons, which is reflected in greater levels of expression of the post-mitotic cellular marker, neuronal nuclear antigen, in smoke-exposed victims (13, 53). Additionally, abnormal levels of neurotransmitters have been shown and abnormal neuronal function due to nicotine exposure (54, 55). These findings can be observed throughout the central nervous system, including the brainstem, cerebellar cortex, and spinal cord, but also in peripheral organ systems (45, 56). Hypoplasia of the pars compacta of the substantia nigra has been observed in SIDS victims; 77% of the SIDS victims had been exposed to maternal smoking; the substantia nigra pars compacta is involved in the sleep arousal phase (57).

## Central Nervous System (CNS)

### Brainstem

Neuropathological studies of both SIUDS and SIDS cases have highlighted both hypoplasia, increased apoptotic figures and abnormal functioning in a number of brainstem nuclei (52, 56, 58). All these abnormalities have been found to be greater following smoking in pregnancy. Many of the affected nuclei are crucial in central respiratory control, for example the pre-Bötzinger complex in the ventral medulla and the pontine Kölliker-Fuse Nucleus (56). Both nuclei play an essential role in respiratory rhythm generation and in coordinating the change between inspiration and expiration (56, 59). The parafacial/facial complex is also required for respiration as it initiates the inspiratory phase of the respiratory cycle in conjunction with the pre-Bötzinger complex (60, 61). Hypoplasia of the parafacial/facial complex occurs predominantly in SIUDS cases, highlighting its importance in the progression to extra-uterine life (48, 56, 62). Further, the dentate-olivary complex and the dentato-rubro-olivary network, which drive the autonomic compensatory respiratory response to episodes of hypoxia, have also been implicated in sudden perinatal death (41, 63). Abnormalities in the arcuate nucleus of the hypothalamus, affecting cardiorespiratory responses and acting as a central chemoreceptor, have been found in > 50% of sudden perinatal death (64). A further vital structure affected in both SIDS and SIUDS is the raphe nucleus, a key producer of serotonin, which can modulate respiratory activity in response to changes in oxygen and carbon dioxide tension and pH levels (54, 65). It has also been proposed to influence intrauterine autonomic nervous system control with respect to vital homeostatic functions (66). Studies have shown a reduction in serotonin throughout the brainstem of sudden perinatal death cases. Nicotine significantly affects alpha 7-nicotinic acetylcholine receptors which have essential roles in the development of the brainstem regions receiving cholinergic projections in perinatal life. High alpha 7-nicotinin acetylcholine receptor expression levels were only observed in one study in the infants of mothers who smoked;, this was frequently associated with hypoplasia of brain structures involved in vital functions (46).

Brainstem nuclei mediating arousal such as the inferior colliculus and locus coeruleus have also been implicated in sudden perinatal death. The inferior colliculus plays an important role in processing auditory information, but also in the sleep-wake cycle (67). The locus coeruleus is the main source of noradrenaline in the brainstem and thereby coordinates multiple autonomic function such as the sleep-wake cycle and cardiorespiratory control (68, 69) and also acts as a central chemoreceptor for carbon dioxide (27, 70). Maternal smoking has been associated with greater rates of hypoplasia and abnormal neurotransmission in the locus coeruleus (68, 69). Specifically, reduced levels of noradrenaline and tyrosine hydroxylase, the enzyme necessary to produce noradrenaline, were observed (68). Nicotine exposure in a primate model produced brainstem and autonomic abnormalities of the key monoamine system that govern the response to hypoxia;. interestingly the effects were offset by coadministration of the anti-oxidant Vitamin C (71). A review has highlighted that the brainstem of infants who died from SIDS exhibited abnormalities in major neurotransmitter and receptor systems including catecholamines, neuropeptides, acetylcholinergic, serotonin, glutamate, brain derived neurotrophic growth factor and cytokines (72).

### Cerebellar Cortex

The cerebellar cortex is particularly vulnerable to exogenous toxins due to its prolonged developmental period, commencing in early fetal life and continuing throughout the 1st year of postnatal life (73, 74). It plays not only a key role in motor coordination, but also the coordination of cardiorespiratory and autonomic nervous systems, with multiple modulatory connections to the brainstem and the cerebral cortex. As such, cerebellar abnormalities can result in dysfunctional breathing following birth, enhancing the risk of sudden death (49). The two main neuronal cell types in the cerebellum, the Purkinje cells and the granule cells, show evidence of hypoplasia and immaturity in sudden perinatal death, with a significant correlation to smoke

exposure. In a study of 21 cases of sudden fetal death and 25 cases of sudden infant death, a high percentage of developmental defects of the purkinje cells were noted (75). Further, abnormal trophic signalling has been evidenced with a significant reduction in Brain-derived neurotrophic factor (BDNF) in the cerebellum of SIUDS/SIDS cases (76). These abnormalities in the developing cerebellar cortex found in SIUDS/SIDS cases were greater following maternal smoking in pregnancy (49).

A particular vulnerable area in the cerebellum to the effects of smoking is the external granule layer (EGL), which is present throughout fetal life, but following birth progressively thins and disappears (73, 74). This area expresses a significant number of nACHRs, highlighting the importance of cholinergic activity in the development and differentiation of EGL neurons. Exposure to tobacco smoke is associated with a significant reduction in the receptor level in sudden perinatal death cases (49).

### Spinal Cord

Spinal cord abnormalities have also been implicated in the pathogenesis of sudden perinatal death and have been shown to be more frequent in infants of mothers who smoked during pregnancy (56). *In utero*, the spinal cord, in particular the intermediolateral nucleus, has been shown to initiate episodic respiratory movements that facilitate lung development (56). Greater rates of hypo-development of this nucleus have been observed in SIUDS and SIDS victims, particularly in infants of mothers who smoked during pregnancy (56, 77).

## Autonomic Nervous System (ANS)

The area postrema plays a key role in chemoreception and controlling autonomic functions. Neuronal hypoplasia, abnormal vasculature and reactive gliosis have been found in this area in both SIUDS and SIDS cases. Importantly, these abnormalities were more common in offspring of smoking mothers (78).

The carotid bodies can also be adversely affected by tobacco exposure. Smoke exposure can lead to abnormal maturation and neurotransmitter levels such as dopamine, leaving infants more vulnerable to hypoxic states (58, 79–81). These findings may further explain the impaired arousal observed in smoke-exposed infants in response to hypoxia (24, 25). A study has demonstrated a significant correlation between unexplained death, altered substance P staining and maternal smoking (82, 83). Another in study, serial sections of 84 brainstems from subjects ranging from 17 weeks gestation to 8 postnatal months of life demonstrated histological and immunohistochemical alterations in the choroid plexus of the fourth ventricle including hyperexpression of substance P and apoptosis (84).

# EFFECTS ON THE RESPIRATORY SYSTEM

Pulmonary hypoplasia has been more frequently observed in animal models exposed to nicotine *in utero*, but not in humans (45, 85). This effect appears to be mediated by exogenous nicotine that leads to an over-expression of nACHRs in the pulmonary epithelium, which leads to abnormal cholinergic activity during lung development (45, 86). In humans, antenatal smoking has an adverse effect on airway development, small airways being affected more than large airways. This is important as such abnormalities are associated with an increased tendency to wheeze in early childhood and predisposes to an enhanced response to an enhanced response to viral infections in early childhood (85). Antenatal exposure to smoking, however, does not result in increased bronchial hyper-reactivity (85). Pulmonary function testing has not demonstrated that infant lung volume is affected by antenatal smoking exposure, other than due to the expected effects of smoking on somatic growth (85). Large epidemiological studies have demonstrated that antenatal smoking exposure increases wheezing in infancy (87) and in the first 2 years after birth (88). In school age children an increased risk of episodes of dyspnoea (89) and an increased risk of asthma and persistent wheezing up to the age of 15 years (90) have been reported. Increased numbers of nACHRs have been observed in fibroblasts in the airways and the pulmonary vasculature (45). In the airways, greater collagen deposition can lead to greater airway resistance. Additionally, increased fibroblast activation by exogenous nicotine in the vasculature can explain the greater rates of pulmonary hypertension and atherosclerosis in smoke-exposed infants (91–93). Respiratory neural network abnormalities, in particular of the arcuate nucleus, were frequently observed in SIUDS cases (86).

# FURTHER CONSIDERATIONS

It remains difficult to predict which fetus or infant will be affected by sudden death. As an important risk factor, reduction in maternal smoking should remain a key focus in trying to reduce rates of sudden fetal or infant death. Anti-smoking laws, such as prohibiting smoking in public places, however, have not resulted in a significant decrease in sudden fetal or infant death (94). Furthermore, nicotine-replacement therapies do not significantly reduce maternal smoking rates (19). Importantly, nicotine replacement therapies still expose the developing fetus to nicotine and thus could be associated with an increase the risk of sudden perinatal death (15, 18). It is therefore, important to educate children regarding the harmful effects of smoking and which may prevent young women from starting smoking.

# CONTRIBUTION TO THE FIELD STATEMENT

Maternal smoking is a risk factor for sudden infant death syndrome (SIDS) and sudden intrauterine unexplained death syndrome (SIUDS). Disappointingly then the global prevalence of smoking during pregnancy is 1.7% and up to 8.1% of women in Europe smoke during pregnancy; 250 million women smoke during pregnancy worldwide. A literature review has been carried out to identify the mechanisms by which antenatal smoking may result in SIDS or SIUDS. This has demonstrated that infants born to mothers who smoke have an abnormal response to hypoxia and hypercarbia and they also have reduced arousal responses. The harmful effects of tobacco smoke are mainly mediated by release of carbon monoxide and nicotine which result in abnormalities in brainstem nuclei crucial to respiratory control, the cerebral cortex, and the autonomic nervous system.

Furthermore, abnormal maturation and transmitter levels in the carotid bodies have been described which would make infants more vulnerable to hypoxic challenges. Sadly, smoking cessation programs appear not to have significantly reduced the number of pregnant women who smoke.

## AUTHOR CONTRIBUTIONS

NB and AG: undertook independent literature reviews. NB: wrote the first draft. All authors were involved in critical review of the literature, production of the manuscript, and approved the final version of the manuscript.

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# Loss of MeCP2 Function Across Several Neuronal Populations Impairs Breathing Response to Acute Hypoxia

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Rett Syndrome (RTT) is a neurodevelopmental disorder caused by loss of function of the transcriptional regulator Methyl-CpG-Binding Protein 2 (MeCP2). In addition to the characteristic loss of hand function and spoken language after the first year of life, people with RTT also have a variety of physiological and autonomic abnormalities including disrupted breathing rhythms characterized by bouts of hyperventilation and an increased frequency of apnea. These breathing abnormalities, that likely involve alterations in both the circuitry underlying respiratory pace making and those underlying breathing response to environmental stimuli, may underlie the sudden unexpected death seen in a significant fraction of people with RTT. In fact, mice lacking MeCP2 function exhibit abnormal breathing rate response to acute hypoxia and maintain a persistently elevated breathing rate rather than showing typical hypoxic ventilatory decline that can be observed among their wild-type littermates. Using genetic and pharmacological tools to better understand the course of this abnormal hypoxic breathing rate response and the neurons driving it, we learned that the abnormal hypoxic breathing response is acquired as the animals mature, and that MeCP2 function is required within excitatory, inhibitory, and modulatory populations for a normal hypoxic breathing rate response. Furthermore, mice lacking MeCP2 exhibit decreased hypoxia-induced neuronal activity within the nucleus tractus solitarius of the dorsal medulla. Overall, these data provide insight into the neurons driving the circuit dysfunction that leads to breathing abnormalities upon loss of MeCP2. The discovery that combined dysfunction across multiple neuronal populations contributes to breathing dysfunction may provide insight into sudden unexpected death in RTT.

**Keywords: Rett, MeCP2, hypoxia, sudden death, biomarker, breathing abnormalities, genetic manipulation, pharmacological manipulation**

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## INTRODUCTION

Rett syndrome (RTT, OMIM 312750) is a neurodevelopment disorder caused by mutations in the X-linked gene *Methyl-CpG Binding Protein 2* (*MECP2*) that is characterized by initial normal development followed by regression manifesting as loss of acquired skills (1–3). Many affected individuals also develop autonomic and physiological deficits including disrupted breathing with bouts of hyperventilation and apnea (4, 5). Approximately one quarter of all deaths in RTT are sudden and unexpected, raising the possibility that breathing abnormalities contribute to some of these deaths (6). The abnormal breathing pattern may indicate deficits in the networks that maintain normal respiratory rhythmogenesis as well as those that modify its response to environmental stimuli (7).

Several mouse models have been generated that display features similar to the human disorder (8–11). While female mice heterozygous for *Mecp2* mutations show many features reminiscent of the human disorder, the nature of the X-linked mutation also causes them to be mosaic for MeCP2 and less severely affected than hemizygous male animals. For these reasons, male mice are often used to help dissect anatomic and neuronal population effects of MeCP2 expression. Mice lacking MeCP2 function exhibit an abnormal response to hypoxia (12–14). Normally during acute hypoxia blood oxygen status is sensed by the carotid body located along the carotid bifurcation and relayed to brainstem respiratory centers through the nucleus tractus solitarius (NTS) (15–17). The expected response to an acute hypoxic challenge in adult mammals is an immediate increase in ventilation in an effort to increase gas exchange and blood oxygenation, within a timescale of minutes, ventilation then decreases as the failure to improve blood oxygen saturation persists and conservation of the remaining oxygen for essential body functions becomes critical (16–19). The increase in ventilation followed by ventilatory decline until ventilation approaches baseline levels is commonly referred to as the hypoxic ventilatory response, with the ventilatory decline stage often referred to as hypoxic ventilatory decline (HVD) (16, 20, 21).

However, in animals lacking Mecp2, the breathing rate during acute hypoxia remains elevated relative to that of wildtype littermates (12, 14, 22). In combination with disordered breathing characterized by bouts of hyperventilation interspersed with apnea, the persistent increase in ventilation in a hypoxic environment may be maladaptive and creates risk from increased metabolic demand without sufficient supply of oxygen (23). Furthermore, the maladaptive reflexive breathing response to external stimuli observed in these animals may reflect the underlying circuit-level disruption that might contribute ultimately to sudden unexpected death. A key unanswered question is the neuronal cell-type contribution to the abnormal hypoxia response in MeCP2 mutant animals. To investigate the underlying etiology of the abnormal HVD that occurs in mice lacking MeCP2, we used a combination of genetic and pharmacological methods to dissect the cellular contribution to the HVD. We identified that mice lacking MeCP2 acquire a deficit in HVD, presenting with a persistently increased breathing rate during exposure to hypoxia. Furthermore, we identified that MeCP2 function within excitatory, inhibitory, and modulatory neuronal populations is critical for a normal HVD.

## MATERIALS AND METHODS

### Animals Used in Experiments

All research and animal care procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and housed in the Association for Assessment and Accreditation of Laboratory Animal Care-approved animal facility at Baylor College of Medicine. $Mecp2^{Tm1Bird}$ mice were obtained as a gift from Dr. Adrian Bird (University of Edinburgh, Edinburgh, UK, RRID:IMSR_JAX_007177) and backcrossed and maintained on a 129S6 background for >10 generations (8). $Mecp2^{Tm1.1Bird}$ mice were generated via Cre mediated recombination of $Mecp2^{Tm1Bird}$ mice and maintained on a 129S6 background. $Mecp2^{Tm2Bird}$ mice were obtained from The Jackson Laboratory and maintained on a C57BL/6J background (RRID:IMSR_JAX_006849) (9). $Mecp2^{TM1.1Bird}$ mice used for experiments were generated by crossing the congenic 129S6 $Mecp2^{TM1.1Bird/+}$ female mice to wild-type male C57BL/6J mice to generate male 129B6F1 $Mecp2^{+/Y}$ (WT) and $Mecp2^{Tm1.1Bird/Y}$ (NULL) mice, and female $Mecp2^{+/+}$ (WT) and $Mecp2^{Tm1.1Bird/+}$ (HET) mice. *Nestin-Cre* (RRID:IMSR_JAX_003771) mice were maintained on a C57BL/6J background; *Th-Cre* (MGI:2677450), and *VIAAT-Cre* (MGI:4867735) and *VGLUT2-Cre* (RRID:IMSR_JAX_016963) mice were maintained on a FVB/NJ strain background. Cre lines were crossed to $Mecp2^{Tm1Bird}$, or $Mecp2^{Tm2Bird}$ to generate male conditional knockout and conditional rescue mice and strain matched control littermates (24–27).

### Unrestrained Whole Body Plethysmography

Unrestrained plethysmography in mice was performed similarly to methods previously described (14, 22). Mice were placed within unrestrained whole-body plethysmography chambers (Buxco), ∼500 mL in volume with a continuous flow rate of 500 mL/min flushing the chambers with fresh air. Mice were allowed to acclimate for at least 20 min, and baseline breathing was then recorded for 30 min. Data were collected during each animals' session within the plethysmograph chambers without additional pre-data collection habituation sessions. To determine response to hypoxic gas (10% $O_2$, balance $N_2$), the chamber was then flushed with hypoxic gas for 20 min. Breathing rate was determined using a customized algorithm written in Matlab (RRID:SCR_001622) using the plethysmography signal files captured with Ponemah3 software (RRID:SCR_017107). To reduce the artifacts from excessive movement and sniffing behavior, breaths that exhibited an inspiratory time <0.03 s, an expiratory time >10 s, and a calculated exhaled tidal volume >150 or <50% of calculated inhaled tidal volume were excluded; breaths were then filtered with intervals excluded if they possessed >10% of their breaths above 500 breaths per minute (sliding 200 breath windows centered on the current breath). The peak breathing rate during hypoxia was calculated as the 90th percentile for breaths during the first 5 min of hypoxic challenge.

Breathing parameters for each animal during baseline and as well as during the declined phase of hypoxic challenge (at 10 min from the onset of hypoxia until 15 min) were determined as the average instantaneous value over the recorded interval and then averaged across trials for animals subjected to repeated measurements. Additional parameters were collected during the baseline portion and include apnea index (apneas per 10,000 breaths, defined as a breath >0.5 s in duration and > 2x the overall baseline breath duration and >2x the duration of the 6 surrounding breaths), irregularity score calculated from breath duration changes (|[n]-[$n_{-1}$]|/[$n_{-1}$]), and uncompensated tidal volume calculated from the box flow of air into and out of the plethysmograph chamber. Respiratory parameters were then compared across genotypes, sex, or treatment as described in the text.

## Pharmacological Manipulation of Hypoxic Breathing Response

Adult male 129B6F1 WT and NULL mice were treated with sterile phosphate buffered saline or drugs dissolved in sterile phosphate buffered saline by intraperitoneal injection immediately preceding plethysmography recordings of the hypoxic breathing response as described above. Following a minimum 48 h "wash-out" period, mice were tested with another drug and dosage combination. Drug and doses used include tiagabine at 3, 10, and 30 mg/kg; muscimol at 1, 2, and 3 mg/kg; baclofen at 1, 2, and 3 mg/kg; desipramine at 3, 10, and 30 mg/kg; ketamine at 10 and 30 mg/kg; and L-DOPA at 30, 60, or 100 mg/kg administered alone or with carbidopa at 10 mg/kg.

## Histological Quantification of Neuronal Activity

Quantification of hypoxia induced neuronal activity and regions selected for analysis were adapted from methods used to look at hypoxia induced activity in the rat brain (28). To reduce artifacts of novelty and stress induced neuronal activation, male mice were habituated to the unrestrained whole body plethysmography chambers for at least 1 h a day for 4 days before being submitted to a gas challenge. On the day of the gas challenge 129B6F1 WT and NULL mice were placed in the plethysmography chambers and subjected to room air for 30 min to establish baseline breathing, mice were then treated for 3 h with hypoxic gas (10% $O_2$, balance $N_2$) or treated with room air to control for basal neuronal activity. Mice were then immediately anesthetized and processed for immuno-histology.

Male mice were deeply anesthetized by intraperitoneal injection with Avertin at a dose of 0.04 ml/g and then fixed by transcardiac perfusion with PBS followed by 4% paraformaldehyde in PBS. Tissues for histological analysis were harvested and fixed overnight in 4% paraformaldehyde in PBS and cryopreserved by overnight incubations in increasing concentrations of sucrose (up to 30% sucrose). Tissues were embedded in O.C.T. compound (Sakura) and sectioned. Sections were collected for at a thickness of 50 μm and stained as floating sections. Sections were blocked for 1 h in a PBS solution containing 10% serum (matched to the host used for the secondary antibodies) and 0.3% Triton X-100. Neuronal activation was assayed by immune-staining for c-Fos expression with a rabbit-anti-c-Fos antibody diluted 1:500 (RRID:AB_2106783), and a goat-anti-rabbit secondary antibody conjugated to dylight 549 (JaxIR). Primary and secondary antibody incubation was performed in the blocking solution for 12–48 h at 4°C or 6 h at room temperature. Sections were washed between incubations with PBS and 0.05% Triton X-100. If not included in the mounting medium, DAPI was included in the penultimate wash. Sections were mounted with Prolong mounting medium (Invitrogen) and imaged via epifluorescent microscopy (Zeiss M1 with Axiovision software, RRID:SCR_002677).

Quantification of neuronal activation bilaterally across regions in the hindbrain was performed via stereological estimation with an optical dissector excluding the upper surface of the tissue, a slide sampling fraction of 1/3 and an area fractionator of 1/4. Image processing and counting of neurons was performed using ImageJ software (29). NTS, locus coeruleus (LC), and Parabrachial Complex (PB) regions were identified based on their anatomic locations as defined in the Paxinos mouse brain atlas (30). Quantified neurons are expressed as the number of neurons bilaterally present in the region per mouse.

## Statistics

All statistics were performed using SPSS (RRID:SCR_002865) on a PC. Parametric statistics were performed using ANOVA for the indicated factors. For conditions where factor had more than two levels, formalized *post-hoc* testing was performed using Student-Newman-Keuls or Bonferroni correction for multiple comparisons to detect pairwise differences.

# RESULTS

## Loss of MeCP2 Function Impairs the Breathing Rate Response to Hypoxia

Mice lacking MeCP2 (male-NULL and female-HET) exhibit a higher breathing rate during exposure to hypoxia (10% O2, balance N2) than their wild-type littermates (14). We sought to better understand the nature of this abnormal breathing response using unrestrained whole body plethysmography. On a 129B6F1 isogenic strain background, we observed that similar to WT mice, NULL mice show a sharp increase in their breathing rate in response to acute hypoxia; however, NULL mice maintain a persistently elevated breathing rate as opposed to WT mice that exhibit HVD and decrease their breathing rate to near baseline levels as the exposure to hypoxia continues within the first 5 min after initiation of hypoxia (**Figures 1A,B**, **Table 1**).

Prior studies have demonstrated that the onset of apnea and disrupted breathing within this model are acquired during the first few weeks of life, progress across the lifespan of the animal, and can be exacerbated by hypoxic or hypercapnic challenge (31, 32). We sought to determine whether the deficit in HVD also is acquired as the animals age. We quantified HVD by determining the average "declined breathing rate" measured from 10 to 15 min after the onset of hypoxia. We observed normal patterns of HVD in NULL mice relative to control littermates at 3 weeks of age. By 4 weeks of age, NULL mice begin developing

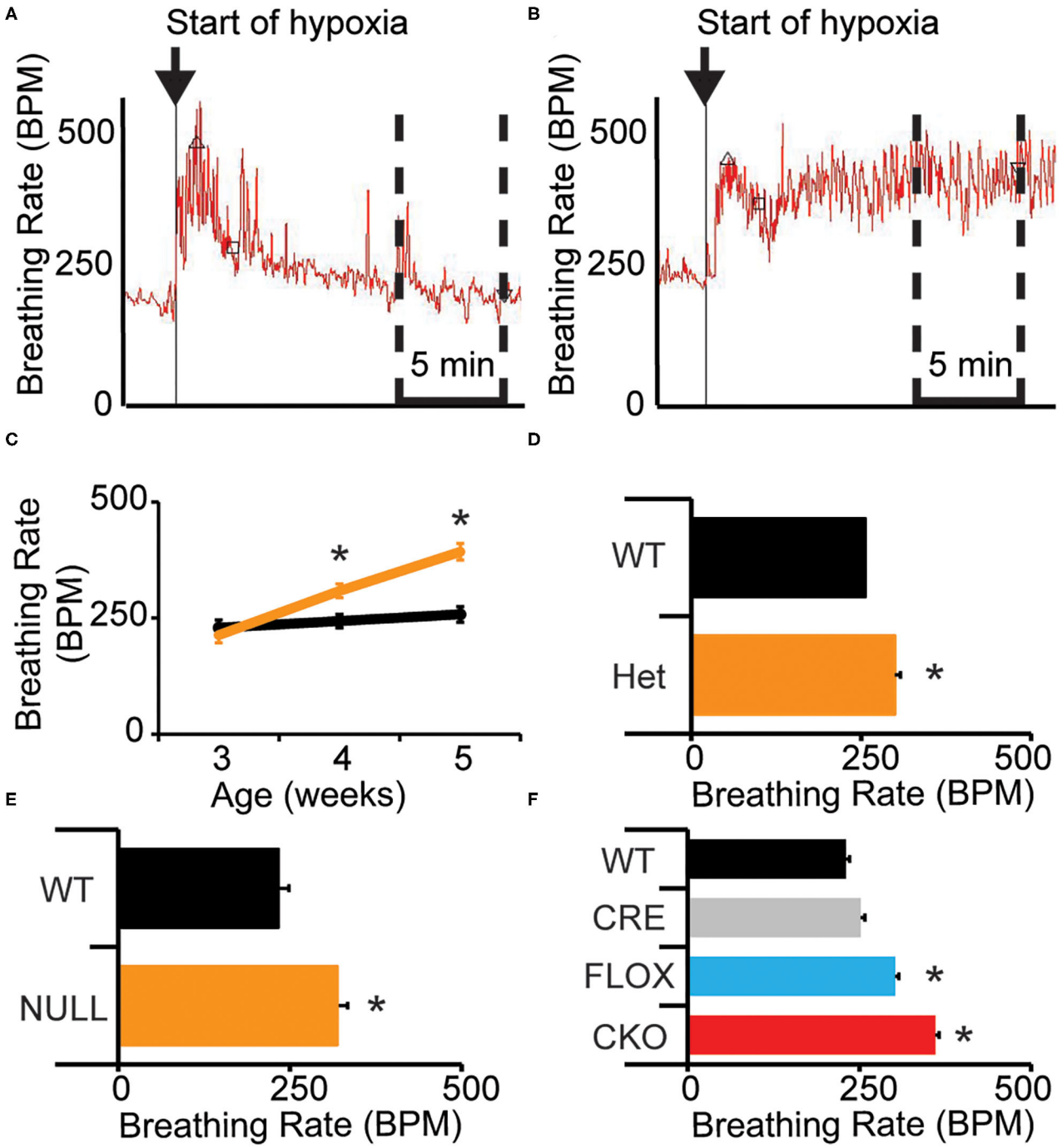


**FIGURE 1 |** Loss of MeCP2 function disrupts the breathing rate response to acute hypoxia. **(A)** Wild-type mice exhibit a typical breathing rate response to exposure to hypoxia, with an immediate increase in breathing rate followed by a decline to near baseline breathing rates—representative image shown. Upward pointed triangle indicates timing of peak hypoxic breathing, defined as the 90th percentile for breathing rate during the first 5 min of hypoxic challenge, square indicates the third minute of the hypoxic challenge. **(B)** NULL mice exhibit an abnormal breathing rate response to hypoxia, with an immediate and persistent increase in breathing rate for the duration of exposure to hypoxia—representative image shown. The time segment used to determine the average "declined breathing rate" between 10 and 15 min after the onset of hypoxia is shown by the dashed box in **(A,B)**. The "declined breathing rate" is presented in **(C–F)**. **(C)** Male 129B6F1 NULL mice acquire the elevated "declined breathing rate" after 3 weeks of age **(D)**. This elevated "declined breathing rate" is also present in aged female HET mice, **(E)** and in male NULL mice on a C57BL/6J background. **(F)** Removal of MeCP2 function from the nervous system with *Nestin-Cre* also causes the elevated breathing rate during hypoxia. **(C–F)** indicate mean +/– S.E.M. *$p < 0.05$ effect of genotype by ANOVA with Student-Newman-Keuls *post-hoc* correction for multiple comparisons for **(F)** (CKO vs. all other genotypes). Number of animals in each group are indicated in **Tables 1**, **2**. Representative plethysmography traces are available (**Supplementary Figures 1, 2**).

**TABLE 1 |** Baseline and hypoxic breathing parameters in *Mecp2* mutant mice grouped by age, strain, and sex.

| Strain | Sex | Age | Genotype | *N* | Baseline BPM^ | 10% O2 Peak BPM | 10% O2 Declined BPM+^ |
|---|---|---|---|---|---|---|---|
| 129B6F1 | Male | 3 wk | WT | 13 | 255.54 ± 32.81 | 479.08 ± 98.78 | 229.14 ± 41.36 |
| | | | NULL | 12 | 243.19 ± 25.73 | 460.82 ± 45.18 | 213.75 ± 74.83 |
| | | 4 wk | WT | 8 | 240.74 ± 12.71 | 494.00 ± 61.13 | 243.96 ± 24.03 |
| | | | NULL | 8 | 271.32 ± 20.82* | 447.55 ± 33.48 | 308.58 ± 53.47* |
| | | 5 wk | WT | 7 | 237.05 ± 10.10 | 504.73 ± 59.67 | 258.04 ± 39.14 |
| | | | NULL | 6 | 260.22 ± 18.58* | 470.79 ± 36.29 | 399.10 ± 36.11* |
| 129B6F1 | Female | 2 yr | WT | 7 | 240.39 ± 17.65 | 484.56 ± 46.72 | 255.87 ± 28.50 |
| | | | HET | 8 | 296.92 ± 38.55* | 430.07 ± 35.58* | 299.59 ± 16.36* |
| C57BL/6J | Male | 2 mo | WT | 10 | 279.24 ± 68.88 | 585.32 ± 32.56 | 233.01 ± 22.71 |
| | | | NULL | 11 | 287.00 ± 32.96 | 516.48 ± 91.96* | 318.92 ± 77.95* |

*Mean breath per minute (BPM) values are presented, +/– SD. *p < 0.05 effect of genotype (within each age, strain, and sex group). +p < 0.05 effect of age (Male 129B6F1 only). ^p < 0.05 effect of age*genotype (Male 129B6F1 only) as determined by ANOVA. Additional parameters including Irregularity Score, Apnea Index, and uncompensated Tidal Volume were also quantified (**Supplementary Table 1**). Representative plethysmography traces are available (**Supplementary Figure 1**).*

a significant HVD deficit that continues to worsen at 5 weeks of age. WT mice show similar patterns of HVD across the tested ages (**Figure 1C**, **Table 1**).

We also wanted to examine if this phenotype was robust enough to be observed across sexes and strain backgrounds. On a 129B6F1 strain background, 2 year old female HET mice reproduce the impaired HVD observed in the males (**Figure 1D**, **Table 1**). Similarly, male NULL mice raised on a C57BL/6J strain background also have an increased breathing rate during exposure to hypoxia (**Figure 1E**, **Table 1**). The trend toward a slight decrease in the peak breathing rate during hypoxic exposure observed in the 129B6F1 male NULL mice, is significant for the female HET mice and C57BL/6J male NULL mice (**Table 1**).

## MeCP2 Function in Excitatory, Inhibitory, and Modulatory Neurons Critical for the Breathing Rate Response to Hypoxia

To better define in which neuronal cell-types MeCP2 expression is required for a normal HVD, we performed genetic removal and restoration of *Mecp2*. This produced wild-type (WT) mice, mice possessing just the *Cre* alleles (CRE), mice possessing just the $Mecp2^{Tm1Bird}$ allele (FLOX) or double mutant mice possessing both *Cre* and $Mecp2^{Tm1Bird}$ alleles (conditional knock out—CKO). The CKO mice are deficient for MeCP2 activity within the cell populations that expressed the cre-recombinase. For genetic rescue studies, we generated WT and CRE mice as well as mice possessing just the $Mecp2^{Tm2Bird}$ allele (STOP) or both *Cre* and $Mecp2^{Tm2Bird}$ alleles (RESC). The STOP mice do not express MeCP2 due to a Cre excisable STOP cassette within the endogenous *Mecp2* locus, while RESC mice have MeCP2 activity solely restored to cells that have expressed the cre-recombinase.

CKO mice produced with Nestin-Cre, lack MeCP2 function within the nervous system and reproduce the impaired HVD observed in the NULL animals (**Figure 1F**, **Table 2**). Additionally, CKO mice with MeCP2 function removed from excitatory (VGLUT2-CKO), inhibitory (VIAAT-CKO) and modulatory neuronal populations (TH-CKO) all show significant impairment in HVD (**Figures 2A,C,E**, **Table 2**). Furthermore, RESC mice with MeCP2 function restored to excitatory (VGLUT2-RESC), inhibitory (VIAAT-RESC), and modulatory (TH-RESC) neuronal populations show significant improvement of their HVD relative to their STOP littermates (**Figures 2B,D,F**, **Table 2**).

## Pharmacologic Manipulation of Excitatory, Inhibitory, and Modulatory Systems Affects the Breathing Rate Response to Hypoxia

Genetic manipulation of MeCP2 function within the excitatory, inhibitory, and modulatory populations demonstrated the critical requirement of MeCP2 function in these neuronal populations for normal HVD. To extend our understanding we pharmacologically manipulated these neurotransmitters to determine if the HVD could be improved within NULL mice. Previous work has shown that loss of MeCP2 function causes a cell-autonomous reduction of expression of the biosynthetic enzymes required for the synthesis of these specific neurotransmitters (25, 26, 33); therefore, we tested whether compounds that enhance signaling of these neurotransmitters (TH neurons: L-DOPA, carbidopa, desipramine; VIAAT neurons: tiagabine, baclofen, muscimol) would modulate the HVD. Additionally we tested ketamine, an NMDA receptor agonist, which has been reported to correct hyperexcitability within forebrain regions of mice lacking MeCP2 (34).

Treatment with L-DOPA bypasses the biosynthetic deficit in the production of dopamine and norepinephrine that is present in NULL mice due to decreased Tyrosine Hydroxylase expression (26). Efficacy of L-DOPA treatment can be improved by co-administration with carbidopa, a structural analog of L-DOPA that inhibits DOPA decarboxylase present in the gut, allowing more L-DOPA in peripheral circulation to cross the blood brain barrier where it can improve noradrenergic and dopaminergic neuron function. Low doses of L-DOPA (30 and 60 mg/kg) had little impact on HVD. However, increased levels of L-DOPA (100 mg/kg) decreased the breathing rate following several minutes of hypoxic exposure (**Figure 3**, **Table 3**). Carbidopa improved

**TABLE 2 |** Baseline and hypoxic breathing parameters from *Mecp2* conditional knockout and conditional rescue mice.

| CKO/CR | Cre | Genotype | *N* | Baseline BPM | 10% O2 Peak BPM | 10% O2 Declined BPM |
|---|---|---|---|---|---|---|
| CKO | Nestin | W | 11 | 217.39 ± 41.73 | 464.07 ± 79.87 | 230.23 ± 30.91 |
| | | C | 9 | 233.48 ± 35.36 | 495.70 ± 80.45 | 251.81 ± 38.92 |
| | | F | 11 | 256.67 ± 43.43 | 517.61 ± 44.85 | 301.87 ± 55.38* |
| | | K | 13 | 307.59 ± 52.13* | 482.24 ± 53.72 | 360.97 ± 63.10* |
| | Vglut2 | W | 13 | 246.61 ± 47.30 | 539.80 ± 28.46 | 297.66 ± 50.85 |
| | | C | 15 | 239.39 ± 33.31 | 524.35 ± 24.36 | 295.67 ± 51.18 |
| | | F | 14 | 264.06 ± 44.39 | 542.15 ± 26.40 | 286.42 ± 28.71 |
| | | K | 11 | 293.19 ± 30.52 | 503.16 ± 17.60* | 415.00 ± 18.42* |
| | Th1 | W | 8 | 219.34 ± 14.79 | 499.32 ± 60.94 | 257.22 ± 39.04 |
| | | C | 7 | 223.88 ± 34.53 | 549.64 ± 43.61 | 276.71 ± 36.10 |
| | | F | 8 | 237.20 ± 24.44 | 530.36 ± 61.40 | 297.02 ± 14.70 |
| | | K | 11 | 226.38 ± 34.37 | 538.47 ± 39.43 | 325.28 ± 15.42* |
| | Viaat | W | 8 | 264.12 ± 55.73 | 520.36 ± 47.03 | 284.30 ± 21.17 |
| | | C | 5 | 236.23 ± 38.06 | 497.89 ± 50.76 | 242.84 ± 26.54 |
| | | F | 7 | 273.85 ± 66.86 | 503.83 ± 53.57 | 272.89 ± 40.04 |
| | | K | 6 | 240.39 ± 23.17 | 472.51 ± 44.40 | 331.26 ± 39.22* |
| CR | Vglut2 | W | 6 | 219.58 ± 22.79 | 536.62 ± 48.76 | 240.16 ± 49.81 |
| | | C | 9 | 250.67 ± 53.49 | 531.37 ± 72.05 | 234.40 ± 31.19 |
| | | S | 5 | 299.98 ± 74.80 | 509.45 ± 61.14 | 357.19 ± 64.80* |
| | | R | 8 | 231.93 ± 34.34 | 551.62 ± 82.17 | 276.94 ± 42.65 |
| | Th1 | W | 6 | 249.82 ± 58.56 | 516.36 ± 40.77 | 255.52 ± 26.31 |
| | | C | 3 | 244.30 ± 47.40 | 418.97 ± 64.24 | 264.37 ± 42.32 |
| | | S | 7 | 251.28 ± 27.80 | 496.28 ± 44.88 | 372.93 ± 50.63* |
| | | R | 8 | 273.19 ± 61.56 | 460.77 ± 28.07 | 287.83 ± 41.26 |
| | Viaat | W | 9 | 255.52 ± 45.10 | 468.35 ± 144.79 | 241.69 ± 28.07 |
| | | C | 9 | 256.36 ± 43.87 | 462.65 ± 130.45 | 253.06 ± 35.02 |
| | | S | 7 | 273.17 ± 31.29 | 489.74 ± 38.37 | 392.88 ± 28.93* |
| | | R | 16 | 238.98 ± 44.85 | 530.35 ± 91.10 | 285.06 ± 38.56 |

*Mean breath per minute (BPM) values are presented, +/– SD. *p < 0.05 effect of genotype for marked groups vs. all other groups in the same Cre-CKO/CR experiment, as determined by ANOVA and Student-Newman-Keuls post-hoc correction for multiple testing. Abbreviations, W, WT; C, CRE; F, FLOX; K, CKO; S, STOP; R, RESC. Additional parameters including Irregularity Score, Apnea Index, and uncompensated Tidal Volume were also quantified (**Supplementary Table 2**). Representative plethysmography traces are available (**Supplementary Figure 2**).*

the response and reduced the dosage of L-DOPA required to modify the breathing rate during hypoxia; however, treatment with high doses of L-DOPA with carbidopa also diminished the peak breathing rate during hypoxia (**Figure 3**, **Table 3**).

Desipramine, a tricyclic antidepressant that inhibits reuptake of norepinephrine, has previously been tested in NULL mice and improves the incidence of apnea caused by loss of MeCP2 (13, 35). Treatment with desipramine (30 mg/kg) significantly improved HVD of NULL mice, with minimal impact on their peak breathing rate during hypoxia (**Figure 3**, **Table 3**).

Treatment of mice with tiagabine, a GABA reuptake inhibitor, improved HVD in NULL animals to levels closer to those of saline treated WT mice (**Figure 3**, **Table 3**). Similarly, the $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen also improved the HVD of NULL animals. However, the dosages of muscimol and baclofen that showed significant reduction in breathing rate during exposure to hypoxia also caused significant decreases in the peak breathing rate during hypoxia. Dosages of tiagabine that improved HVD had less impact than muscimol or baclofen on the peak breathing rate during hypoxia (**Figure 3**, **Table 3**).

Both dosages of Ketamine that were tested showed no improvement in HVD, the higher 30 mg/kg dose trended toward a worsening of the impaired HVD ($p = 0.01$ ANOVA for effect of drug on NULL mice, not corrected for multiple comparisons) (**Figure 3**, **Table 3**).

While some treatments, such as L-DOPA without carbidopa, desipramine, baclofen, and ketamine, had little to no impact on the hypoxic breathing response in WT animals, several others such as L-DOPA with carbidopa, tiagabine, and muscimol also modified the WT hypoxic breathing response.

## Mice Lacking MeCP2 Function Have Decreased Hypoxia Induced Neuronal Activity

Finally, we wanted to determine if loss of MeCP2 modified hypoxia induced neuronal activation as an explanation of the

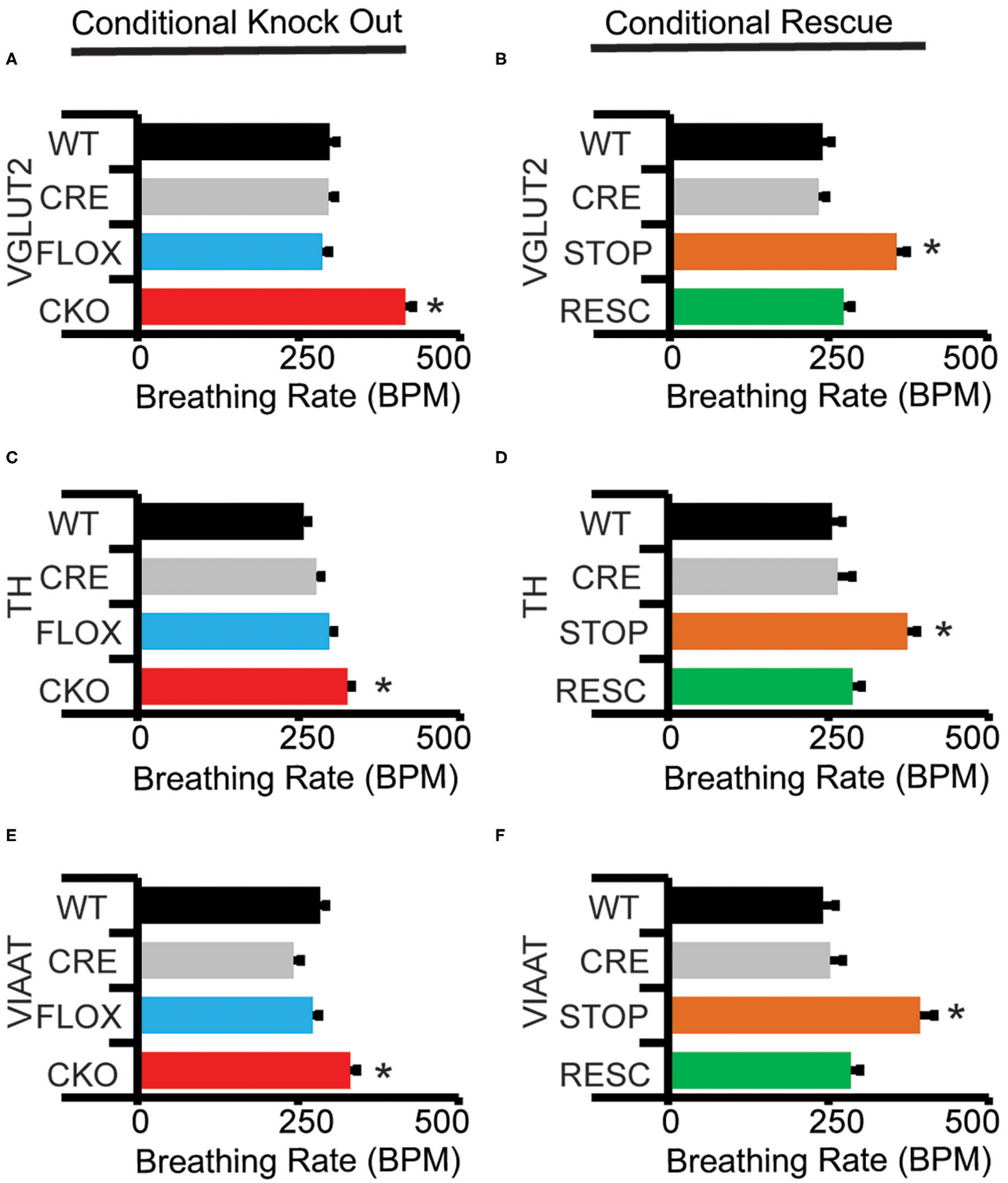


**FIGURE 2 |** Conditional knock-out and conditional rescue of MeCP2 shows necessity and sufficiency of MeCP2 expression for hypoxic breathing decline. "Declined breathing rate" (between 10 and 15 min after the onset of hypoxia) data is presented for genetic conditional experiments generated with **(A,B)** *VGLUT2-Cre*, **(C,D)** *TH1-Cre*, and **(E,F)** *VIAAT-Cre*. Values indicate mean +/− S.E.M. *$p < 0.05$ effect of genotype by ANOVA with Student-Newman-Keuls *post-hoc* correction for multiple comparisons (indicated genotype vs. all other genotypes). ns not significant. Number of animals in each group is presented in **Table 2**. Representative plethysmography traces are available (**Supplementary Figure 2**).

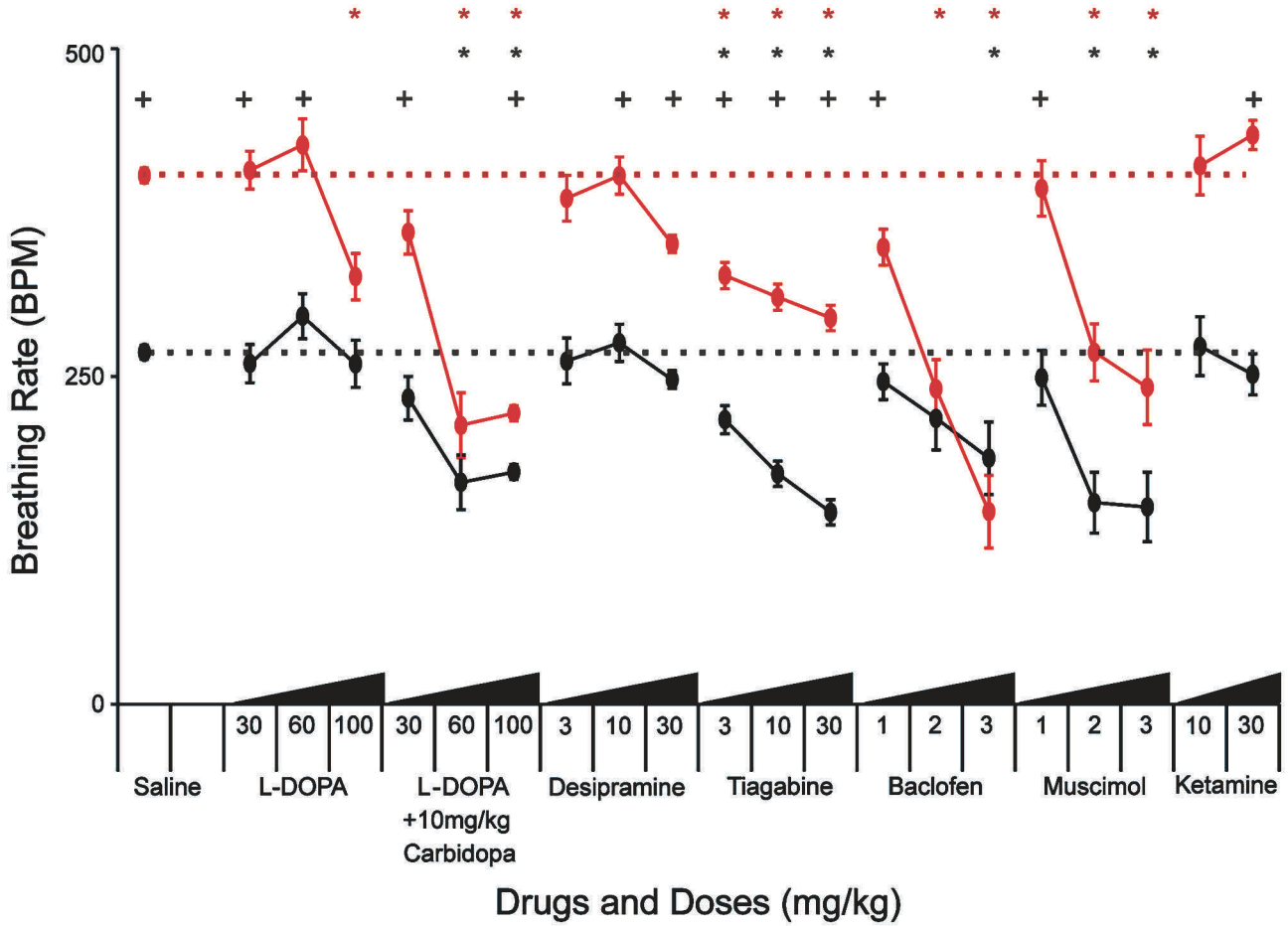


**FIGURE 3 |** Pharmacological treatments modify breathing rate during acute hypoxia. "Declined breathing rate" (between 10 and 15 min after the onset of hypoxia) data is presented for 129B6F1 mice treated with saline and various concentrations of the indicated drugs 1 h before exposure to hypoxia. Values indicate mean +/– S.E.M. *$p < 0.05$ effect of drug vs. saline on breathing rate within genotype (black-WT, orange-NULL). +$p < 0.05$ effect of genotype within the indicated drug and dose. *p*-values determined by ANOVA with Bonferroni *post-hoc* correction for 61 multiple comparisons (40 for effect of drug vs. saline and 21 for effect of genotype at each drug and dose). Number of animals in each group is presented in **Table 3**.

abnormal HVD. Hypoxia induced neuronal activation was assayed by immunostaining for c-Fos reactivity in mice that were treated with hypoxic gas compared to mice allowed by continue breathing room air. We performed stereological quantification of c-Fos immunoreactivity in regions that are activated by exposure to hypoxia including the LC, PB, and NTS. All three anatomic regions showed an increase in c-Fos positive nuclei in both WT and in NULL animals; however, NULL mice had a 20% deficit in hypoxia induced c-Fos expressing nuclei in the NTS (**Figure 4**).

## DISCUSSION

MeCP2 is required for an appropriate breathing rate response to acute hypoxic challenge. Genetic knockout and rescue of *Mecp2* within glutamatergic, GABAergic, and dopaminergic/noradrenergic neurons indicates that MeCP2 function is required in a distributed set of neurons for a normal breathing response to hypoxia. Genetic knockout and rescue of *Mecp2* within the caudal pons and medulla (using *HoxB1*-Cre) indicated that the critical neural circuitry for the normal HVD is within these brain regions (14, 22). In combination, this indicates that a neural circuit within the caudal pons and medulla comprised of excitatory, inhibitory, and dopaminergic/noradrenergic neurons underlie the HVD. Because within this region there are no dopaminergic neurons, it is likely that the MeCP2 requirement for normal HVD in TH-expressing neurons is solely within the noradrenergic neurons.

Other studies identified an increase in resting activity, as measured by c-Fos staining, within the NTS of NULL mice relative to WT controls; our current experiment is underpowered to confirm this in the room air condition (34). However, upon exposure to hypoxia, there is a clear increase in c-Fos expression in both genotypes, consistent with studies looking at brain regions activated by exposure to hypoxia (28). Interestingly,

**TABLE 3 |** Baseline and hypoxic breathing parameters following acute drug treatment.

| Drug | Dose mg/kg | Genotype | *N* | Baseline BPM | 10% O2 Peak BPM | 10% O2 Declined BPM |
|---|---|---|---|---|---|---|
| Saline | | WT | 73 | 224.05 ± 32.61 | 495.13 ± 66.98 | 268.31 ± 47.62 |
| | | NULL | 78 | 268.11 ± 36.67+ | 488.02 ± 39.95 | 403.26 ± 46.53+ |
| L-DOPA | 30 | WT | 8 | 200.73 ± 9.13 | 440.01 ± 82.86 | 259.72 ± 29.84 |
| | 60 | WT | 6 | 243.66 ± 33.19 | 514.42 ± 56.40 | 295.82 ± 70.39 |
| | 100 | WT | 8 | 227.36 ± 10.04 | 411.19 ± 71.78* | 259.53 ± 35.82 |
| | 30 | NULL | 8 | 260.29 ± 21.86+ | 478.68 ± 56.41 | 407.29 ± 50.06+ |
| | 60 | NULL | 8 | 296.08 ± 36.12 | 501.84 ± 20.95 | 426.62 ± 22.63+ |
| | 100 | NULL | 8 | 266.57 ± 45.23 | 427.66 ± 55.62 | 326.09 ± 61.47* |
| L-DOPA + 10mg/kg Carbidopa | 30 | WT | 12 | 251.99 ± 38.75 | 444.94 ± 71.41 | 233.39 ± 57.62 |
| | 60 | WT | 5 | 205.61 ± 21.63 | 338.77 ± 88.41* | 169.07 ± 37.00* |
| | 100 | WT | 34 | 204.61 ± 28.59* | 324.14 ± 48.14* | 177.01 ± 26.05* |
| | 30 | NULL | 12 | 334.14 ± 22.04*+ | 492.57 ± 35.02 | 359.78 ± 57.40+ |
| | 60 | NULL | 7 | 244.11 ± 100.76 | 340.40 ± 111.44* | 212.76 ± 64.47* |
| | 100 | NULL | 36 | 219.16 ± 44.74* | 312.25 ± 48.87* | 221.92 ± 39.24*+ |
| Desipramine | 3 | WT | 4 | 199.18 ± 23.82 | 507.59 ± 70.93 | 261.73 ± 33.77 |
| | 10 | WT | 4 | 206.82 ± 14.09 | 529.00 ± 72.21 | 275.64 ± 29.45 |
| | 30 | WT | 6 | 219.48 ± 12.72 | 420.68 ± 35.47 | 247.50 ± 14.73 |
| | 3 | NULL | 4 | 266.09 ± 58.38 | 469.60 ± 22.87 | 385.83 ± 36.08 |
| | 10 | NULL | 4 | 289.39 ± 33.26 | 516.58 ± 33.67 | 403.16 ± 27.52+ |
| | 30 | NULL | 6 | 326.75 ± 67.87* | 470.95 ± 22.90 | 351.02 ± 17.90+ |
| Tiagabine | 3 | WT | 22 | 194.53 ± 12.25* | 430.29 ± 55.97* | 216.92 ± 48.17* |
| | 10 | WT | 15 | 189.07 ± 18.67* | 376.44 ± 51.35* | 175.68 ± 19.50* |
| | 30 | WT | 4 | 256.41 ± 16.18 | 357.73 ± 16.94* | 146.38 ± 21.06* |
| | 3 | NULL | 20 | 289.96 ± 28.97+ | 460.32 ± 30.31 | 326.91 ± 47.03*+ |
| | 10 | NULL | 16 | 282.39 ± 30.76+ | 472.42 ± 56.26+ | 310.40 ± 49.91*+ |
| | 30 | NULL | 4 | 250.37 ± 23.00 | 465.69 ± 18.79+ | 294.49 ± 17.80*+ |
| Baclofen | 1 | WT | 8 | 200.67 ± 16.42 | 537.31 ± 39.75 | 245.95 ± 39.75 |
| | 2 | WT | 7 | 195.51 ± 11.63 | 517.88 ± 34.51 | 218.01 ± 39.98 |
| | 3 | WT | 4 | 204.46 ± 4.26 | 396.02 ± 86.35 | 187.62 ± 54.11* |
| | 1 | NULL | 8 | 209.96 ± 26.95* | 446.94 ± 29.74+ | 348.51 ± 37.86+ |
| | 2 | NULL | 6 | 237.42 ± 44.36 | 350.07 ± 78.26*+ | 240.36 ± 75.99* |
| | 3 | NULL | 4 | 143.58 ± 35.03* | 197.42 ± 14.49* | 146.77 ± 56.90* |
| Muscimol | 1 | WT | 8 | 217.09 ± 9.16 | 495.59 ± 73.38 | 248.87 ± 34.29 |
| | 2 | WT | 8 | 171.61 ± 30.81* | 387.34 ± 86.31* | 153.70 ± 66.62* |
| | 3 | WT | 7 | 148.70 ± 49.41* | 396.39 ± 71.50* | 150.34 ± 32.11* |
| | 1 | NULL | 8 | 277.90 ± 54.56 | 507.23 ± 87.24 | 393.38 ± 76.74+ |
| | 2 | NULL | 7 | 234.31 ± 20.17+ | 365.48 ± 68.62* | 268.45 ± 54.91* |
| | 3 | NULL | 8 | 188.31 ± 36.68* | 370.08 ± 38.41* | 241.65 ± 97.85* |
| Ketamine | 10 | WT | 4 | 241.09 ± 15.72 | 501.78 ± 53.76 | 272.85 ± 22.41 |
| | 30 | WT | 4 | 223.04 ± 15.88 | 510.12 ± 83.80 | 251.58 ± 25.55 |
| | 10 | NULL | 4 | 282.47 ± 40.02 | 482.83 ± 53.74 | 410.80 ± 59.30 |
| | 30 | NULL | 2 | 292.04 ± 14.25 | 513.16 ± 18.61 | 434.15 ± 5.37+ |

*Mean breath per minute (BPM) values are presented, +/– SD. *$p < 0.05$ effect of drug versus saline. +$p < 0.05$ effect of genotype within the same drug and dosage as determined by ANOVA with Bonferroni post-hoc correction for 61 multiple comparisons (40 for effect of drug vs. saline and 21 for effect of genotype at each drug and dose). Additional parameters including Irregularity Score, Apnea Index, and uncompensated Tidal Volume were also quantified (**Supplementary Table 3**).*

NULL mice have 20% fewer c-FOS expressing neurons within the NTS compared to WT controls. The c-FOS activation within the LC and PB did not show an effect size sufficient to be detected; however, the limited power of the present study prevents a definitive conclusion that these regions are not impacted in NULL mice. The impairment in hypoxia induced neuronal activation within the dorsal medulla's NTS, a key relay center in the processing of hypoxic stimuli, thus becomes an attractive candidate for contributions to the MeCP2 related impairment in HVD observed in NULL animals. The role for the NTS is also

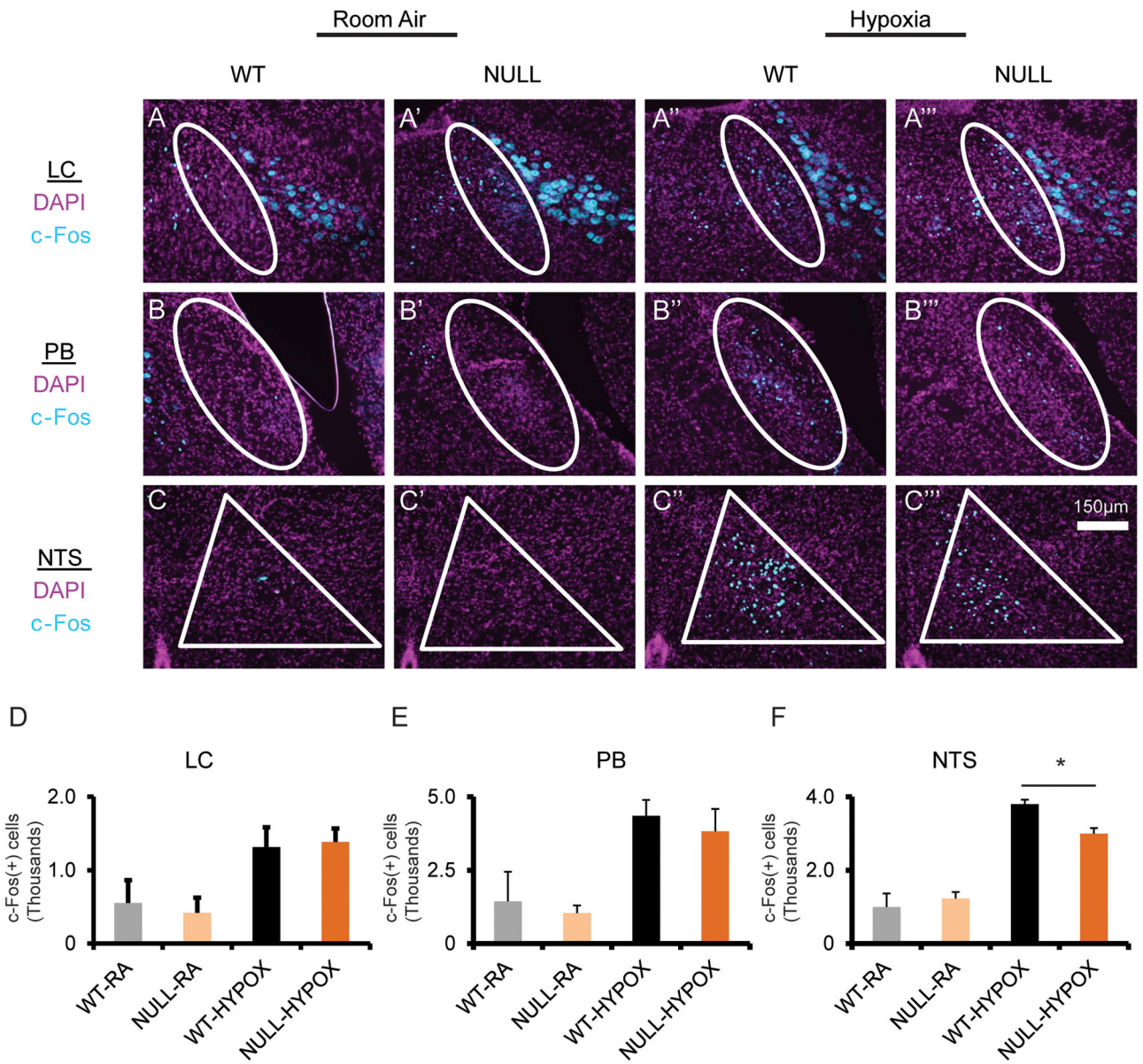


**FIGURE 4 |** Mice lacking MeCP2 function exhibit decreased hypoxia induced neuronal activation. Treatment with hypoxia (10% O2, balance N2) for 3 h induces neuronal activation across several brainstem regions, as determined by immunofluorescent staining for c-Fos expression in coronal sections through the brainstem containing the locus coeruleus (LC) **(A–A''')**, Parabrachial Complex (PB) **(B–B''')**, and nucleus solitary tract (NTS) **(C–C''')**. Stereological quantification of c-Fos expressing nuclei identified an increase in neuronal activation in both WT and NULL mice during treatment with hypoxia in the LC **(D)**, PB **(E)**, and NTS **(F)**. However, the quantity of neurons activated in the NTS of NULL mice was 20% lower than WT littermates (Room Air treated WT $N = 2$, Room Air treated NULL $N = 2$, Hypoxia treated WT $N = 3$, Hypoxia treated NULL $N = 3$). Indicated brain regions outlined in white. $^*p < 0.05$ for indicated comparison by One-Way ANOVA with Student-Newman-Keuls *post-hoc* correction for multiple comparisons. RA, room air; HYPOX, hypoxia. Bar graphs indicate mean values, error bars indicate SEM.

supported by previous anatomically focused conditional genetic studies which suggest key centers mediating the aberrant MeCP2 dependent hypoxic breathing phenotype do not extend beyond the rostral boundary of developmental expression of HoxB1 or HoxA4 (14, 22).

Excitatory/inhibitory balance within the NTS contributes to HVD (36). Inhibition of NMDA receptors within the NTS is thought to play a part in HVD, particularly via PDGF-beta signaling. This is due to observation of impaired HVD in mice heterozygous for a mutation in the PDGF-beta receptor as well as in mice lacking PDGF-beta expression in the brain (37, 38). Other studies have been able to demonstrate that treatment with the NMDA receptor agonist ketamine can ameliorate several phenotypes present in mice lacking MeCP2 including disrupted breathing and apnea (34, 39). We were unable to replicate the breathing improvement from ketamine treatment, potentially

due to the limited number of animals tested, as well as to differences in dose and acute treatment vs. chronic dosing used in prior studies (**Supplementary Table 3**) (39). Similarly, we did not see any trend toward improvement of HVD from ketamine treatment.

Glutamatergic release in the NTS stimulated by hypoxia provides a pool of glutamate available for conversion to GABA and use for inhibitory signaling. The requirement of MeCP2 for GABA biosynthesis and GABAergic neuronal function suggest this as a likely contributor to the abnormal HVD observed in *Mecp2* mutant mice (25). Consistent with this hypothesis, modulation of GABAergic function within the NTS of rats by microinjection with GABA agonists and antagonists modulates the ventilatory response to hypoxia (36).

The tyrosine hydroxylase neurons within the central nervous system also are involved in HVD, as blocking dopamine activity with haloperidol prevents HVD from occurring (40). Furthermore, A2/C2 adrenergic neurons within the NTS begin to lose expression of TH in the absence of MeCP2 by 1 month of age, correlating with the onset of the abnormal HVD observed in *Mecp2* mutant mice (33). Optogenetic stimulation of C2 TH neurons genetically deficient for *VGLUT2* suggest that the TH and VGLUT2 populations overlap and that TH dependent modulation of breathing is dependent on VGLUT2 expression (41).

The neuronal circuits that control the breathing response to hypoxia span multiple anatomic regions, and categories of neurons. The initially normal HVD observed in young NULL mice implies that MeCP2 function is not essential to the establishment of these circuits, but that it is critical across multiple components for their maintenance as the deficit manifests by 4 weeks of age. Pharmacological treatment of NULL mice targeting excitatory, inhibitory, and modulatory neurons suggests that the impairment in HVD is due to decreased activity across each of the populations.

While not the main objective of the current study, we were also able to monitor additional breathing parameters of baseline breathing including apnea and irregularity of breathing (**Supplementary Tables 1–3**). Overall, we did observe an expected increase in the incidence of apnea in NULL mice relative to WT animals as well as effects and trends indicating increased breathing irregularity scores in NULL mice. These non HVD outcome measures presented with large variation making definitive conclusions from the genetic and pharmacological studies underpowered with respect to apnea and breathing irregularity. However, we did note that some of the pharmacological treatments, such as L-DOPA with carbidopa, baclofen, and muscimol, modified breathing under sustained hypoxia and resulted in worsening of apnea as well as irregularity. Interestingly, these treatments also blunted the peak breathing response to hypoxia while tiagabine and desipramine reduced the breathing rate during sustained hypoxia while sparing the peak breathing response to hypoxia and did not appear to aggravate the incidence of apnea or breathing irregularity.

These experiments attempted to dissect the neuronal substrates modified by the absence of MeCP2 and result in impaired HVD. In this sense, the impaired HVD can be considered another biomarker used as an indicator for neurological impairment due to the loss of MeCP2. Our findings are consistent with the pleiotropic action of MeCP2, and with the distributed nature of the brainstem circuitry that regulates HVD (22), and provide insight into the neuronal cell and circuit basis of breathing abnormalities in RTT. As these breathing abnormalities may underlie the sudden and unexpected death seen in RTT, dissecting the pathophysiology and developing targeted therapeutic interventions may be able to help prevent these deaths.

It is important to note that while the experiments looking to dissect the HVD impairment were largely performed using male mice, we did observe the phenotype in female HET animals. Among the key differences between male and female animals with *Mecp2* mutations is the mosaicism of MeCP2 expression within the neuronal populations under study due to *Mecp2* being X-linked and subject to X-chromosome inactivation. Other studies have contrasted the effects of genetic studies between the male and female models. Typically, the phenotypes observed in the female models are less severe due to their mosaicism, but differential benefit may be observed depending on the system that is targeted for genetic restoration (24, 27). Specifically, this contrast is apparent within the genetic rescue of MeCP2 within the excitatory and inhibitory neuronal populations performed by Meng et al. and Ure et. al.; female mice showed greater benefit from rescue in the excitatory neurons while male mice showed greater benefit from rescue in the inhibitory neurons (24, 27). This effect is likely due to activity imbalances within their respective networks upon restoration of MeCP2 with network components expressing varying degrees of mosaicism. Overall, these and previous findings are encouraging regarding development of potential therapies for RTT because they provide additional evidence of neuronal circuits that remain present but are poorly tuned in the absence of MeCP2. Pharmacological or genetic therapies have the potential to re-adjust these circuits to a more normal state; however, full rescue will likely require broad acting interventions that target multiple neuronal populations to achieve balanced activity.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Baylor College of Medicine Institutional Animal Care and Use Committee.

## AUTHOR CONTRIBUTIONS

CW, T-WH, and JN contributed to conception and design of the study. CW, T-WH, JH, DP, and EA performed experiments and collected data. CW and CM developed analysis tools, CW analyzed the data. CM, RS, AI-I, XM, KU, and HZ, contributed to the genetic studies of *Vglut2, Viaat, and Th* conditional

knock-out and rescue. CW and JN wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.593554/full#supplementary-material

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# Immunohistochemical Expression of the Alpha Nicotinic Acetylcholine Receptor 7 in the Human Normal, Diabetic, and Preeclamptic Placenta and Products of Conception

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Preeclampsia (PE) and gestational diabetes (GD) are complications in advanced pregnancy while miscarriage for early pregnancy. However, the etiological factors are not well understood. Smoking has been associated with these complications as well as the sudden intrauterine deaths, sudden infant death, miscarriages, and still births. However, the immunolocalization of alpha 7 nicotine acetylcholine receptor (α7-nAChR) is not studied.

**Materials and Methods:** α7-nAChR subunit expression was evaluated in 10 paraffin-embedded placental tissues after delivery and 10 tissue samples of products of conception during first trimester by immunohistochemistry. Among the placental tissues, two samples were normal placental tissue, four from PE mother, and four from GD mother. The expression of α7-nAChR was compared between the two groups in general and within the subgroups of placenta as well. Protein expression was evaluated using the nuclear labeling index (%) of villi with positive cells stained, positive cells in the decidua, and intensity of staining in the outer villous trophoblast layer.

**Results:** The expression of α7-nAChR protein was high in all the cases of placenta and products of conception (POCs). α7-nAChR expression showed no notable differences among different cases of miscarriages irrespective of the mother's age and gestational age at which the event occurred. However, there were some changes among the normal, PE, and GD placental groups in the linings of the blood vessels. Changes were restricted to the villi (as opposed to the decidua) lining cells, both cytotrophoblast and syncytiotrophoblast, and were specific to the α7 subunit. PE blood vessel lining was thicker and showed more expression of this receptor in endothelial cells and myofibroblasts in PE and GD groups. In POCs, the strong expression was observed

in the decidua myocytes of maternal blood vessels and in syncytiotrophoblast and cytotrophoblast of chronic villi.

**Conclusion:** Nicotine acetyl choline receptors are found to be expressed highly in the placental tissues and in products of conception. They may be associated with the sudden perinatal deaths and miscarriages or complications of pregnancy.

**Keywords: placenta, products of conception, nicotine receptors, alpha 7 acetylcholine receptor, sudden perinatal deaths**

## INTRODUCTION

The prevalence of preeclampsia (PE) and gestational diabetes (GD) during pregnancy is 2–8% (Umesawa and Kobashi, 2017), respectively. The hazardous effects of exposure to tobacco smoking, whether active or passive, during pregnancy include placenta previa (Shobeiri and Jenabi, 2017), placental abruption (Ananth et al., 2016), ectopic pregnancy, early membrane rupture, low birth weight (Bar-Zeev and Solt, 2018), premature birth, sudden perinatal deaths, and stillbirths (Lavezzi et al., 2017; Anderson et al., 2019). We have highlighted these aspects in previous studies as well (Lavezzi et al., 2010, 2011, 2020; Mehboob, 2017; Mehboob et al., 2017; Muhammad et al., 2018).

Tobacco smoke contains many neurotoxic ingredients, but nicotine is the one with highest adverse effects on neurotransmission such as cholinergic system during the development of central nervous system in fetus (Klenowski and Tapper, 2018). Acetylcholine (ACh) is the main cholinergic neurotransmitter and has a basic functional role in the development of nervous system via its synaptic mechanisms of its nicotinic Ach receptors (nAChs) (Brown, 2019). There are nine nAChR subunits: α2, α3, α4, α5, α7, α9, β1, β2, and δ. Nicotine can mimic the effect of ACh and incorrectly signal the cholinergic system to activate when there is no requirement (Robbins, 2016).

Particularly, the α7 subunit affects the developing nervous system in a toxic way and damages the neuronal differentiation, angiogenesis, axon formation, and synaptic transmission (Cross et al., 2017; Lavezzi, 2018). This phenomenon highlights the vulnerability of α7-nAChRs as potential targets for other neurotoxicants, apart from nicotine, during the critical developmental phases of brain. A study was conducted to test this hypothesis, and it was observed that chlorpyrifos, an organophosphate pesticide used in agriculture, exhibits similar actions to those of nicotine in stimulating α7-nAChRs (del Pino et al., 2016). Previous studies have indicated that α2– 7-, 9, 10-, and β1-nAChR subunits are localized in the placenta (Ghazavi et al., 2013; Machaalani et al., 2014, 2018)) but with varying expressions depending on the structure and type of cell. However, a study that is detailed and in different populations has not been conducted yet.

Hence, the current study aimed to evaluate the immunohistochemical expression and localization of α7-nAChRs in three groups of placenta (normal, preeclamptic, and gestational diabetic) after delivery and retained products of conception (POCs) after miscarriage in the first trimester. The purpose is to assess the association with miscarriages and sudden perinatal deaths and explore the involvement of pesticides and smoking exposure in developing countries like Pakistan.

## MATERIALS AND METHODS

### Tissue Collection

Ten placental tissue samples, comprising of two normal, four PE, and four GD samples, were obtained from the Obstetrics and Gynecology Department, after the ethical approval of The University of Lahore Teaching Hospital, Lahore, Pakistan. Placentas were collected within the first hour of delivery. Additionally, the retained POC tissue samples were collected from patients having miscarriages during the first trimester. Four small (2 × 2 cm) separate samples were obtained systematically from different areas of the POCs and placentas. Placental samples were obtained from the maternal side, code side alveoli, fetal side, and necrotic area and allowed to get fixed in 10% formalin for 24 h. Samples were embedded in paraffin and stored at room temperature for sectioning. Sections of 5 μm were cut using a microtome and mounted on 3-aminopropyltriethoxysilane (APES)-coated slides in preparation for staining. Tonsil tissue was used as a positive control for α7-nAChR receptor.

### α7-nAChR Immunohistochemistry

Paraffin-embedded sections were deparaffinized with different grades of ethanol (100–70%), two washes in xylene for 5 min each and three washes in phosphate-buffered saline

**TABLE 1 |** Scoring scale for quantification of staining.

| 0 | 0–1% | Negativity |
|---|---|---|
| + 1 | Less than 10% | Weak positivity |
| +2 | 10–40% | Moderate positivity |
| + 3 | 40% or above of the counted cells | Strong positivity |

**TABLE 2 |** Patients characteristics.

| Characteristics | Placenta (*n* = 10) | POCs (*n* = 10) | *P*-value |
|---|---|---|---|
| No. of cases | 10 | 10 | |
| Maternal age | 33.20 ± 2.57 | 30.40 ± 1.71 | 0.010** |
| Stain score | 3.0 ± 0.0 | 3.0 ± 0.0 | 0.038** |

***Independent sample t-test.*

(PBS) (pH 7.4). Antigen retrieval was carried out by citrate buffer, heated for 60°C. Hydrogen peroxidase was used for blocking the endogenous peroxidase activity for 30 min at 4°C. Non-specificity in the tissue section was blocked by 10% normal rabbit serum and then incubated with the rabbit polyclonal, α7-nAChR primary antibody (Abcam, ab10096, 1:200 dilution) at 4°C overnight. Tissue sections were washed in PBS and incubated with goat antirabbit immunoglobulin G (IgG) secondary antibody (PK-6101, Vector Laboratories, CA, United States). Sections were further processed with the avidin–biotin immunoperoxidase technique (VEDH-4000, Vector Laboratories, CA, United States), counterstained with 3,3′-diaminobenzidine (DAB) and coverslipped.

## Statistical Analysis

Data were analyzed by using SPSS 25.0. All the quantitative variables were presented in mean ± SD. Independent sample t

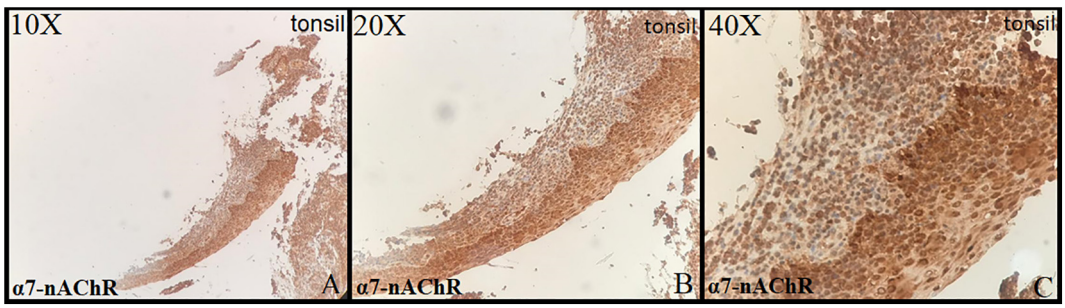


**FIGURE 1 |** Control: tonsil (10×, 20×, 40×) strong positive stain in the squamous cells of lining epithelium (+3 intensity).

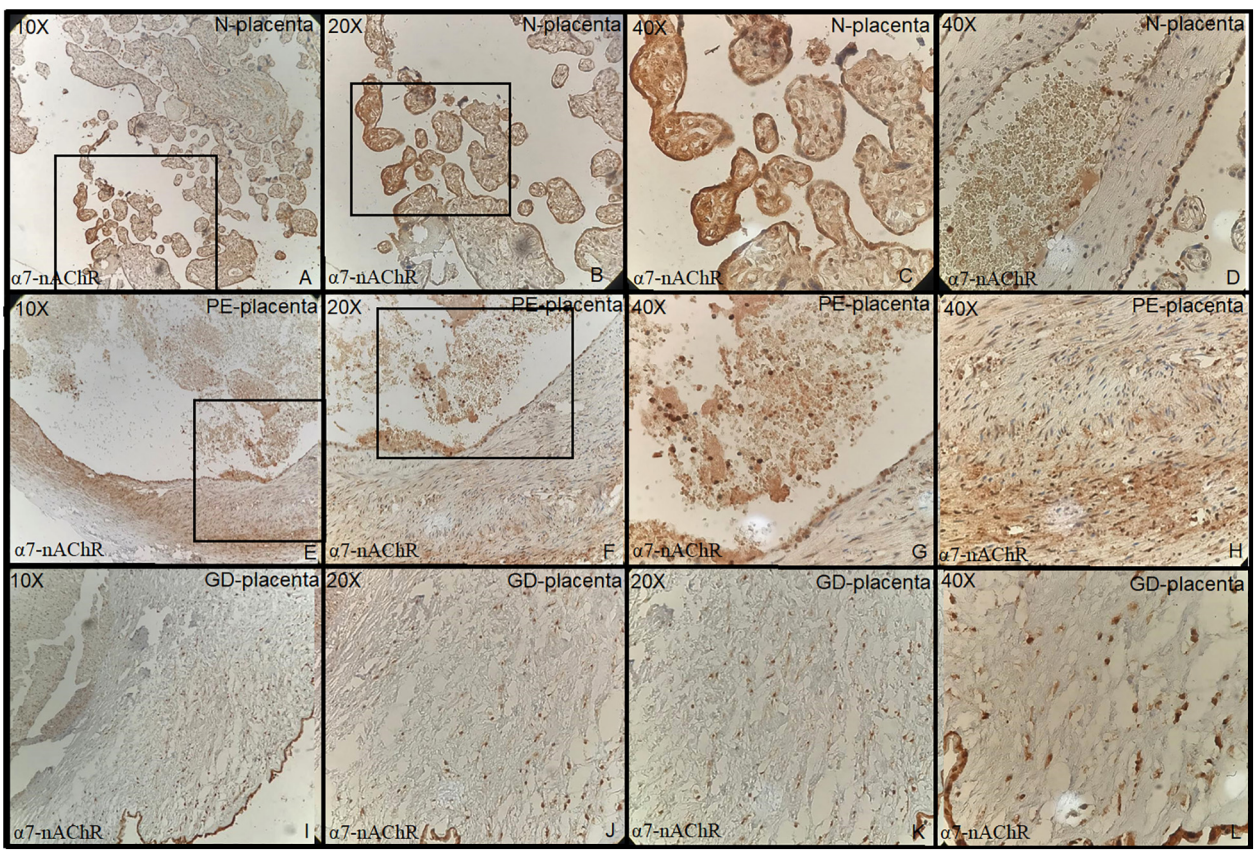


**FIGURE 2 | (A–D)** Normal placenta (10×, 20×, 40×) revealed positive strong staining in chorionic villi lining cells both cytotrophoblast and syncytiotrophoblast. **(E–H)** Preeclampsia: Placenta (10×, 20×, 40×) shows increased thickness of media of the blood vessels walls with positive staining of endothelial cells and myofibroblast. **(I–L)** Gestational diabetes: Placenta revealed strong positivity of endothelial cell and myofibroblast (media is thickened) positive cell line (all + 3 intensity).

test was applied to check the mean difference in both groups (placenta and POCs). $P < 0.05$ was considered as significant.

## α 7-nAChR Expression Quantification

Immunoreactivity in the tissue sections was assessed in each randomly selected nucleus and cell as the number of cells exhibiting a dark brown stain, divided by the total cells, and shown as% [nAChR immunopositivity index (nAChR-I)] (**Table 1**) (Lavezzi et al., 2014).

## Quantitative Analysis for Immunohistochemistry

Images of placenta and POCs were captured using an Olympus BX40 microscope (Artisan Scientific, Champaign, IL, United States) at 10×, 20×, and 40× magnification. The number of cells with positive and negative stain in all the tissue sections was counted by using cell counter function manually.

# RESULTS

## Patient Characteristics

There were 10 cases in the placenta and 10 cases in the POC groups. There were 2 cases in the placenta, 4 in the gestational diabetes, 4 in the pre/eclampsia, and 10 cases in the POC groups. The mean maternal age of patients in Group I was 33.20 ± 2.57 years and that in Group II was 33.20 ± 2.57 years ($P < 0.05$). The mean stain score in each group was 3.0 ± 0.0 ($P > 0.05$; $P < 0.05$) (**Table 2**).

## α7-nAChR Expression in Normal, PE, and GD Placenta and Product of Conception

Tonsillar tissue was strongly positive with + 3 intensity (**Figures 1A–C**). Immunohistochemical expression and localization of nAChR were evaluated and observed to be intense in all the cases of PE and GD as well as in the normal placenta (**Figure 2**) and POCs (**Figure 3**). α7-nAChR showed no significant differences among different cases of miscarriages irrespective of the mother's age and gestational age at which the event occurred. The α7 receptor was widely distributed in the umbilical cord, fetal membranes, and placenta. There was intense staining in the decidual cells, trophoblastic cells of fetal membranes, and chorionic villi (**Figure 2**). The staining was primarily nuclear but also observed in cytoplasmic cells. Within the villi, the expression was highest, with no differentiation among the others (**Figure 2**). The specific staining pattern of α7-nAChR receptor was observed in the cell nucleus in the same decidua maternal villi in which cytoplasmic staining was well marked in syncytiotrophoblast and cytotrophoblast of chronic villi.

# DISCUSSION

The immunohistochemical expression of α7-nAChR subunit in the three groups of placentas (NP, PE, and GD) as well as in POCs showed villi changes specific to α7. The expression was high in the villi and decidua. To date, few studies have been found regarding the immunohistochemical expression of α7-nAChR in the placenta. This includes Lips et al. (2005) who reported the

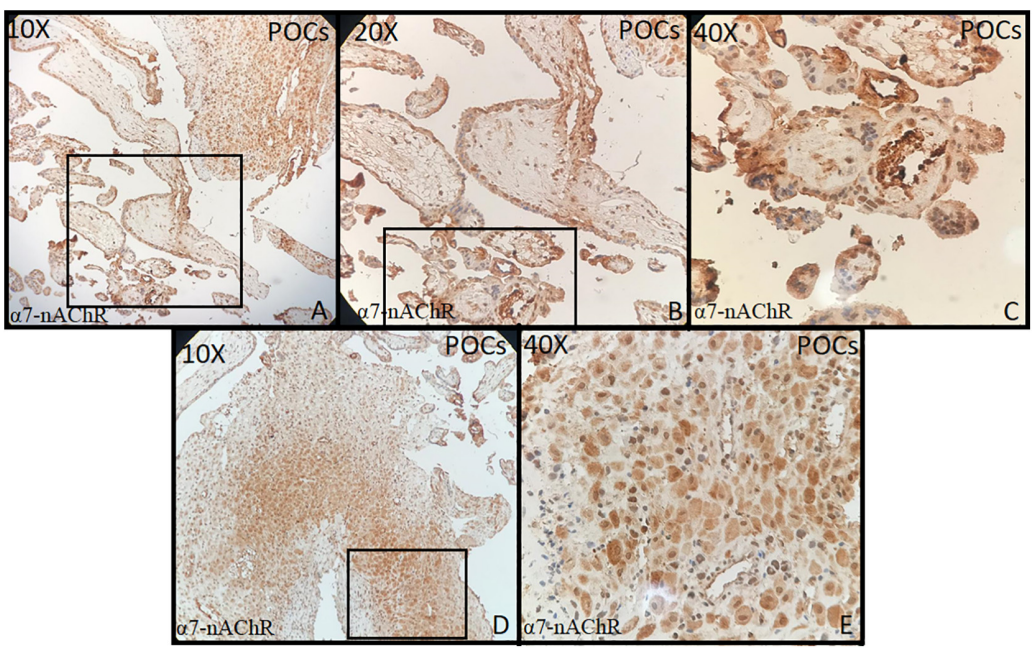


**FIGURE 3 | (A–E)** Products of conception (POCs) (10×, 20×, 40×) showed strong staining in the decidua, endothelial, myocytes of maternal blood vessels, and in syncytiotrophoblast and cytotrophoblast of chronic villi (all + 3 intensity).

nAChR messenger RNA (mRNA) expression; Kwon et al. (2007) who reported α7 expression; and a recent study by Aishah et al. (2017, 2019) who reported the mRNA and protein expression of the α3 and β1 subunits. These studies showed that the nAChR subunits were expressed in the placenta, and their levels vary according to the cellular type. Herein, we report that the α7 subunit was more expressed in the villi and decidua and that they did not differ in the intensity of expression.

The study in 2018 by Machaalani et al. (2018) revealed the highest expression of α4 expression in placenta among the nine subunits studied. In our study, the highest α7 was in the normal placental cells of the villi lining cells both cytotrophoblast and syncytiotrophoblast. Increased thickness of media of the blood vessel walls with positive staining of endothelial cells and myofibroblast were seen in the PE and GD groups as compared to the control group. Small sample size is a limitation in our study.

Here, we have reported the protein localization and its expression in terms of intensity of α7-nAChR subunit in placental tissues and POCs in Pakistani population. The other subunits should also be explored in further studies. Additionally, different geographical locations within Pakistan, as well as in other countries, must also be investigated to see any possible differences. Pakistan is an agricultural country, and many banned pesticides are in use, e.g., dichlorodiphenyltrichloroethane (DDT), organochlorines, and organophophates, which have high nicotine content. Furthermore, cigarette smoking and huqqa smoking by men may put women and unborn child at risk due to passive smoking. This situation vary in different countries and may provide a possible link for diagnosis of at-risk individuals, awareness among society, and a direction for policy makers for banning the hazardous pesticides and smoking at public places.

## CONCLUSION

Immunohistochemical expression and localization was almost similar in all placental groups and POCs. However, some exceptional prominent changes were in the villi in PE and GD groups. There was thickening of the lining of blood vessels in the placenta of these groups, which may be responsible for poor oxygen supply and complications for the fetus. More studies are required to further explore all nicotinic receptors in such cases to find a plausible association with sudden infant deaths, sudden intrauterine deaths, still births, and miscarriages.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Lahore ethical review board. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AA contributed in the planning, write up and designing and facilitated financially and logistically to make this study happen. KE contributed in microscopy and preparing the figures. All the authors have contributed to this study.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Pediatrics

CASE REPORT
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# Case Report: A *de novo* CTNNB1 Nonsense Mutation Associated With Neurodevelopmental Disorder, Retinal Detachment, Polydactyly

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*CTNNB1* gene mutation was firstly reported related to intellectual disability in 2012, to explore the clinical phenotype and genotype characteristics of *CTNNB1* mutation, we collected and analyzed the clinical data of a child with a neurodevelopmental disorder caused by a mutation of *CTNNB1*. The child had dysmorphic features, microcephaly, hypotonia, polydactyly, retinal detachment, and neurodevelopmental disorder, with a *de novo* mutation of *CTNNB1* c.1603C > T, p.R535X. The patient was diagnosed as Neurodevelopmental disorder with spastic diplegia and visual defects (NEDSDV) and was given rehabilitation training. After 4 months of rehabilitation training, she improved in gross motor function. We found that *CTNNB1* mutation can cause neurodevelopmental disorder, which could be accompanied by retinal detachment and polydactyly. The retinal detachment had only been reported in two Asian patients, and we firstly reported the phenotype of polydactyly in the *CTNNB1* mutation. This report not only helps to expand the clinical phenotype spectrum of the *CTNNB1* gene mutation but also prompts a new insight into genetic diagnosis in patients with a neurodevelopmental disorder, retinal detachment, and polydactyly.

**Keywords: CTNNB1 gene, neurodevolpmental disorder, retinal detachment, polydactyly, case report**

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## INTRODUCTION

*CTNNB1* (OMIM: 116806) gene encodes β–catenin protein, which is an integral part of the cadherin/catenin complex and is related to the activation of the Wnt signaling pathway.

It found that β–Catenin knockout mice showed the same behavior as autism spectrum disorder (1), while the loss of function of the *CTNNB1* gene was related to intelligence disorder (2). *CTNNB1* gene mutations have a variety of phenotypes, such as Colorectal cancer, Exudative vitreoretinopathy 7, Hepatocellular carcinoma, Medulloblastoma, Neurodevelopmental disorder with spastic diplegia and visual defects and Pilomatricoma (3). Neurodevelopmental disorder with spastic diplegia and visual defects(NEDSDV, OMIM:615075) was the only one presenting neurological impairment, which was characterized by global developmental delay, impaired intellectual development, axial hypotonia, and dysmorphic craniofacial features with microcephaly. Many patients have visual abnormalities, ranging from strabismus to optic nerve atrophy and retinal abnormalities. Spasticity may also occur in the affected individuals, particularly of the lower extremities, and may have behavioral abnormalities (4).

At present, 33 cases of the disease have been reported in the world (2, 5–11), with only two in Asia (8, 10). This paper reports the clinical characteristics and genetic analysis of the third case of neurodevelopmental disorder caused by *CTNNB1* gene mutation in the Asian population, and we found that polydactyly may be a new feature of *CTNNB1* mutation.

## CASE REPORT

The patient was a 15-month-old girl who came to our department developmental retardation. She is the second child of Chinese parents who are both healthy, young, non-consanguineous. She has a healthy elder brother. During pregnancy, ultrasound scan showed intrauterine growth retardation. She weighed 2.35 kg at birth. The family history was negative for birth defects, developmental delay, intellectual delay, and/or any other neurological disorders.

After a normal neonatal period, she was found with motor retardation when she can't raise her head at 3 months old. At the age of 4 months, she had an ophthalmologic examination and was found blindness in the left eye when her parents wanted to treat strabismus her left eye. Ultrasonic examination showed a retinal detachment in the left eye. Fortunately, her right eye was normal. The patient was evaluated in our department at 15 months. She can raise her head but unstably, and still can't sit. The language barrier is very serious. She can make some sounds, but she can't speak. er parents found her intelligence lower than her peers. Physical examination showed as follows: weight 7.5 kg (−2.46 SD), height 72.5 cm(−2.29 SD), occipitofrontal circumference(OFC) 41 cm (−4.17 SD), anterior frontal was closure, light hair color, fair skin, low set ears, flat nasal bridge, strabismus in the left eye, thin upper lip, polydactyly in the right hand, but the foot does not have polydactylous. The systolic murmur of grade 2/6 can be heard in the 3–4 intercostals of the precordial area. The lung and abdomen are normal. The tension of trunk and peripheral limbs decreased significantly. Bilateral tendon reflexes were weakened, Babbitt's sign was negative, and no sign of spastic paralysis (**Figures 1A–E**). Auxiliary examination: the whole blood count, urine routine test,


**FIGURE 1 | (A,B)** Craniofacial dysmorphism; **(C)** microcephaly; **(D)** polydactyly; **(E)** Hypotonia.

**TABLE 1 |** Clinical findings in our patient and patients reported (4) before with the same mutation.

| | Our patient | Patient 1 | Patient 2 |
|---|---|---|---|
| **Gender** | **Female** | **Male** | **Male** |
| Gestational weeks | 38 | 40 | 41 |
| Birth weight (kg) | 2.35 | 3.05 | 3.4 |
| Maternal age | 24 | 39 | 28 |
| Paternal age | 26 | 43 | 38 |
| Age of onset of symptoms | Birth | 3 months | Birth |
| Age at diagnosis | 1 year 3 months | 3 years 3 months | 14 years |
| Prenatal issues | Intrauterine growth retardation | None | None |
| Neonatal issues | None | Low oxygen saturation | Persistent pulmonary hypertension |
| Growth Parameters (SD) | At 15months: OFC 41 cm (4.17SD ) Weight 7.5 kg (2.46 SD ) Height 72.5 cm (2.29 SD ) | At 21 months: OFC 43 cm (5.25 SD) Weight 10.45 kg (1.29 SD) Height 83.5 cm (0.31 SD) | At 6 years: OFC50.4 cm (1.93 SD) Weight 26 kg (1.17 SD) |
| Microcephaly | Yes | Yes | No |
| Fair skin (ethnic origin) | Yes(Chinese) | Yes (Scottish Caucasian) | Yes (White British) |
| Hair | Light hair color | Very blonde | Fair, not sparse, has a cowlick |
| Dysmorphic features | Low set ears, flat nasal bridge, strabismus in the left eye, thin lip, polydactyly | Thin upper lip; prominent lower lip; long smooth philtrum; small ears; brachycephaly. | Low set ears; short philtrum; thin upper lip; high palate; prominent chin |
| Peripheral spasticity | No | Yes | Yes |
| Truncal hypotonia | Yes | Yes | No |
| Developmental progress | Raised her head at 12 months but still unstably at 15months, still can't sit at age 15months | Smiled at 4 months; Sat supported −8 months; Rolling −9 months; not walking at 3 | Sat at 15 months Walked with rollator—age 4; Can't walk unaided at age 14 years |
| Speech impairment | Makes some noises but no words. | Makes lots of noises but no words. | Single words at age 14 years |
| Visual defect | Strabismus and retinal detachment of left eye | Strabismus and hypermetropia, | None |

biochemical test, thyroid function, and the level of 25 (OH) D3 were all normal. Color Doppler echocardiography showed atrial septal defect, right heart enlargement, pulmonary hypertension, and tricuspid regurgitation (**Table 1**).

Because of her abnormal features (light hair color, fair skin, low ear position, flat nose, strabismus in the left eye, thin lips), microcephaly, hypotonia, development delay, retinal detachment, and polydactyly. Karyotype examination and the whole-exome sequencing were arranged for the patient, and her parents also did the whole-exome sequencing, because the patient's brother was healthy, he didn't take genetic test. Karyotype of the patients was normal, and a mutation c.1603C > T, p.R535X (gene position: CHR3: 41275708-41275708) was on exon 10 of chromosome 3, resulting in the loss of CTNNB1 gene function. The parents do not have the same gene mutation, then the child is identified as a *de novo* mutation (**Figures 2**, **3**).

According to the clinical manifestations and gene mutation, the child was diagnosed as NEDSDV, and rehabilitation training was recommended. After 4 months of rehabilitation training, the child can raise her head steadily, but unable to sit, however, there was no significant improvement in language.

## DISCUSSION

In this study, we present a 15-month-old Chinese girl with a complex phenotype, which included dysmorphic features (light hair color, fair skin, low set ears, flat nasal bridge, strabismus in the left eye, thin lip), microcephaly, hypotonia, development delay, retinal detachment, and polydactyly. WES of the patient revealed a *de novo* heterozygous nonsense mutation in exon 10 of the *CTNNB1* gene (c.1603C > T, p.R535X). This mutation had been reported in only two patients (2). All three patients had serious development delays, especially speech impairment. However, there were still some differences in the phenotype. The polydactyly and retinal detachment reported in our case has not been seen in the two cases previously reported.

*CTNNB1* gene is located on 3q22.1 and encodes β–Catenin protein. Its mutation is related to many diseases, such as a

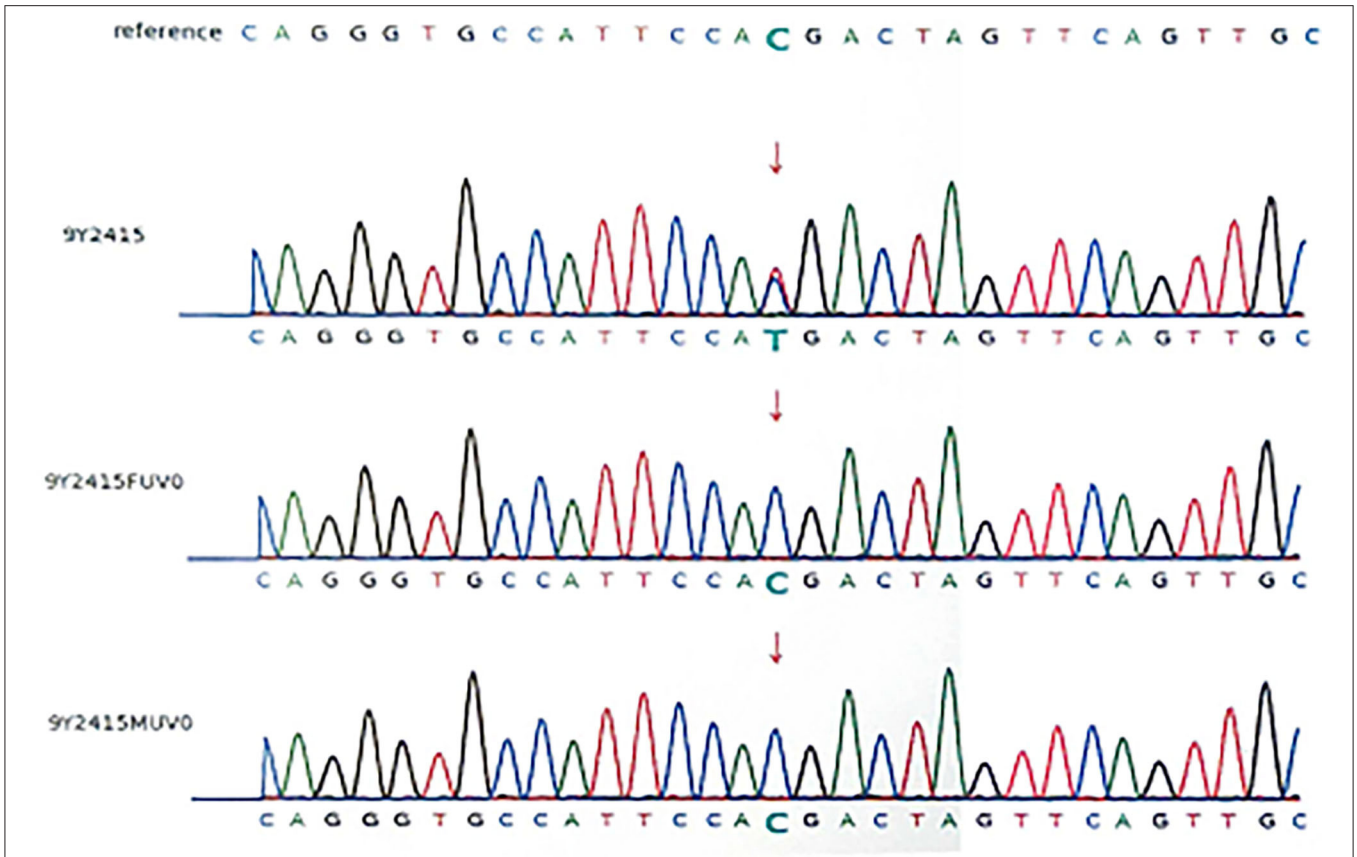


**FIGURE 2 |** DNA electrophoregram with the c.1603C > T,p.R535X mutation in exon 10 of *CTNNB* 1. 9Y2415:patient; 9Y2415FUVO:father; 9Y2415MUVO:mother.

tumor, autism, and so on. In 2012, Joep de ligt et al. reported for the first time that three patients with intellectual disability had *CTNNB1* gene mutation, and their common phenotypes were: severe intellectual disability, absent or very limited speech, microcephaly, and spasticity with a severely impaired ability to walk. So far, 33 cases in the world have been reported. In 2017, the first case from Asian was reported (8), and as far as we knew, our case was the third case of the Asian population suffering from *CTNNB1* mutation related neurodevelopmental disorders.

The patient we reported was similar to the previous reports, but the polydactyly was not reported in the previous cases, and retinal detachment was also rare in the previous case reports, only two cases in the Asian population reported with retinal detachment (8, 10). However, the mutations of the *CTNNB1* were different from each other among the three patients. Previous studies have shown that heterozygous mutations in *CTNNB1* can cause non-syndromic familial exudative vitreoretinopathy(FEVR) and that FEVR was part of the *CTNNB1* haploinsufficiency phenotype (12), but the retinal detachment was only reported in the Asian population. Whether retinal detachment occurs in the Asian population is related to the genetic basis of the population, or because of the bias of too few reported cases, it needs to be further studied. Our patient expresses polydactyly, a unique symptom that has not been seen among other reported patients with *CTNNB1* mutation. Its occurrence may be related to the abnormal Wnt / β signal pathway caused by *CTNNB1* mutation. It has been found that mutation mice with polydactyly showed general de-regulated high levels of canonical Wnt/b-catenin signaling, it hypothesized that these Wnt/b-catenin signaling defects may contribute to the high proliferation rates in organ (13).

To date, 34 cases [one our case and 33 cases reported before (2, 5–11)] were reported. It appears to be a significant gender bias in those affected by inactivating *CTNNB1* mutations(22 females,12 males). Although this is a small sample from which to infer such a finding and that may become more obvious as more cases are identified. Thirty-three lost-function mutations of *CTNNB1* have been reported in the 34 patients, 19 nonsense mutations, nine frameshift mutations, three splice mutations, two complete gene deletions. The mutants were distributed in exons 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, and introns 5, 7, and 10. Among the 34 patients, the mutations of 33 patients were *de novo*, while one patient inherited the mutation (c.734+1G>A) from her mother who had similar but slighter clinical manifestations (10). The mechanism of *CTNNB1* mutation leading to neurodevelopmental delay is not clear, but animal experiment identified that β-catenin loss could result in severe learning impairments, upregulation of γ-catenin (a partial functional homolog, whose neural-specific

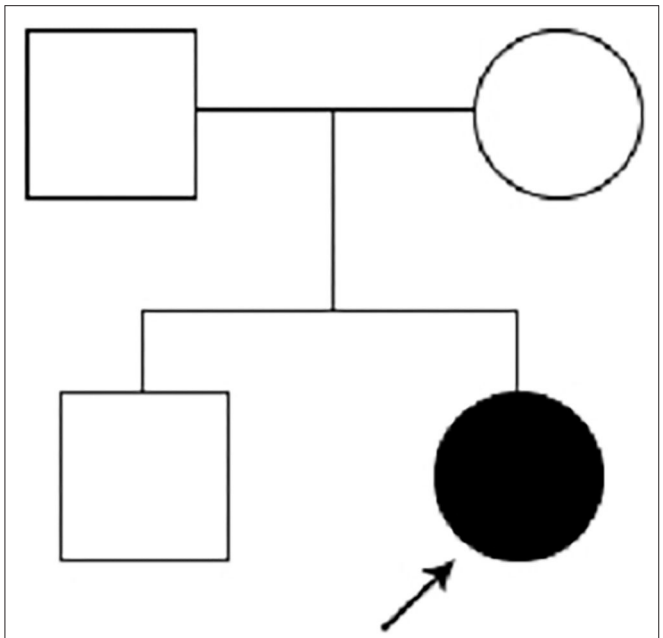

**FIGURE 3 |** Pedigree of the family.

role is poorly defined) and reductions in synaptic adhesion and scaffold proteins which may affect brain development and function (14). Because of poor understanding of the molecular mechanisms of the disease, therapeutic strategies are limited. Most of the patients just were given rehabilitation training and symptomatic treatment. The progress is very poor, all patients suffered severe intellectual disability and can't live by themselves.

In conclusion, our study reported a heterozygous nonsense mutation in the *CTNNB1* gene (c.1603C > T, p.R535X) in a Chinese family with neurodevelopment disorder, retinal detachment, and polydactyly. As far as we know, this is the third case reported in the Asian population and with a special retinal detachment which was only be reported in the Asian population, and we also found that polydactyly may be a new feature of *CTNNB1* mutation. This report not only helps to expand the clinical phenotype spectrum of the *CTNNB1* mutation but also prompts a new insight into genetic diagnosis in patients with a neurodevelopmental disorder, retinal detachment, and polydactyly.

### Limitations and Strengths

We presented the clinical phenotype and genetic variation of a case with *de novo* CTNNB1 Nonsense Mutation, and firstly reported the phenotype of polydactyly in the CTNNB1 mutation. However, there were still some deficiencies, because of insufficient follow-up time, the development and prognosis of the case were unknown.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/ supplementary material.

## ETHICS STATEMENT

Written informed consent was obtained from the mother of the child for the publication of any potentially identifiable images or data included in this article.

## AUTHOR CONTRIBUTIONS

ZK contributed to the acquisition, analysis of data, and writing the first draft. YC contributed to the conception of the work and revised the paper. All authors contributed to the article and approved the submitted version.

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# Revisiting the Neuropathology of Sudden Infant Death Syndrome (SIDS)

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**Background:** Sudden infant death syndrome (SIDS) is one of the leading causes of infant mortality in the United States (US). The extent to which SIDS manifests with an underlying neuropathological mechanism is highly controversial. SIDS correlates with markers of poor prenatal and postnatal care, generally rooted in the lack of access and quality of healthcare endemic to select racial and ethnic groups, and thus can be viewed in the context of health disparities. However, some evidence suggests that at least a subset of SIDS cases may result from a neuropathological mechanism. To explain these issues, a triple-risk hypothesis has been proposed, whereby an underlying biological abnormality in an infant facing an extrinsic risk during a critical developmental period SIDS is hypothesized to occur. Each SIDS decedent is thus thought to have a unique combination of these risk factors leading to their death. This article reviews the neuropathological literature of SIDS and uses machine learning tools to identify distinct subtypes of SIDS decedents based on epidemiological data.

**Methods:** We analyzed US Period Linked Birth/Infant Mortality Files from 1990 to 2017 (excluding 1992–1994). Using t-SNE, an unsupervised machine learning dimensionality reduction algorithm, we identified clusters of SIDS decedents. Following identification of these groups, we identified changes in the rates of SIDS at the state level and across three countries.

**Results:** Through t-SNE and distance based statistical analysis, we identified three groups of SIDS decedents, each with a unique peak age of death. Within the US, SIDS is geographically heterogeneous. Following this, we found low birth weight and normal birth weight SIDS rates have not been equally impacted by implementation of clinical guidelines. We show that across countries with different levels of cultural heterogeneity, reduction in SIDS rates has also been distinct between decedents with low vs. normal birth weight.

**Conclusions:** Different epidemiological and extrinsic risk factors exist based on the three unique SIDS groups we identified with t-SNE and distance based statistical measurements. Clinical guidelines have not equally impacted the groups, and normal birth weight infants comprise more of the cases of SIDS even though low birth weight infants have a higher SIDS rate.

**Keywords: sudden infant death syndrome (SIDS), neuropathology, cluster analysis, infant mortality rate, infant death**

## INTRODUCTION

Few topics in forensics enwrap themselves in as much controversy as the neuropathology associated with sudden infant death syndrome (SIDS). The interaction of concurrent epidemiological risks often associated with impoverished communities adds a level of complexity to SIDS pathogenesis (1, 2). As SIDS correlates with other markers of poor prenatal and postnatal care, which are generally rooted in the lack of access and low quality of healthcare endemic to impoverished racial and ethnic groups, some have come to view SIDS as a disease of health disparities (3–5). Although these epidemiological associations are undeniable, there is also a compelling case, at least in a subset of SIDS decedents, for a primarily neurological, and potentially neuroanatomical, etiology to the patient's death (6). This has significant impact on health policies since they would require implementation of different solutions to prevent these tragedies. For instance, can SIDS be prevented by abrogating health disparities across society, or should investment in basic, mechanistic research into the biological basis of SIDS be prioritized so that we may identify medical interventions? We posit that these policies would not represent mutually exclusive mechanisms to SIDS etiology. Attempts to determine SIDS events due to neurological mechanisms will emerge as a major research need within forensic neuropathology and may complement existing measures in prevention that have been successful in reducing the incidence of SIDS (7, 8).

SIDS neuropathological research has primarily focused on evaluation of the brainstem, due to its critical role in the regulation of the autonomic nervous system. In 1976, Dr. Neaye identified reactive astrogliosis in the medullary reticular formation in association with SIDS (9). Reactive gliosis often represents a general (often chronic) response to a brain injury and, depending on the context, induces protective or detrimental pathways to modulate neurological function. Although astrocytic gene expression changes occur within 1 hour post-brain injury, morphological changes of astrogliosis manifest along more sub-acute time frames (10). In rodents, middle cerebral artery occlusion induces morphological changes in astrocytes first detectable 2 days post infarction with additional dynamic morphological changes for 14 days (11). Thus, morphologically detectable brainstem astrogliosis in a SIDS decedent may imply a subacute/chronic developmental neuropathological change in an otherwise healthy infant, rather than an acute event such as an accidental asphyxiation. However, it is yet to be determined whether brainstem reactive astrogliosis indicates a primary abnormal developmental neuropathological event or is secondary to hypoxia-ischemia (9, 12, 13). With identification of brainstem gliosis, researchers have extensively focused on brainstem nuclei and their projections to other brain regions that have critical roles in autonomic regulation.

The brainstem serotonergic (5-HT) system regulates respiration and airway patency, homeostatic functions, sleep and arousal (12). The medullary 5-HT system is divided into rostral and caudal domains with projections distributed widely throughout the central nervous system. The rostral 5-HT domain projects to telencephalic and diencephalic structures responsible for cognition and arousal. In contrast, the caudal 5-HT domain projects primarily to effector nuclei in the rhombencephalon such as the nucleus of the solitary tract, pre-Bötzinger complex, phrenic nucleus, hypoglossal nucleus, dorsal motor nucleus of the vagus, and nucleus ambiguus. These nuclei integrate diverse, yet interrelated functions including thermoregulation, cardiovascular homeostasis, inspiratory rhythmogenesis, respiratory rhythm generation, diaphragmatic innervation, and airway patency during sleep. It is hypothesized that these disruptions in the brainstem 5-HT system are due to a combination of genetic and environmental factors. In the event that an infant with an inherent underlying vulnerability during this critical period faces an exogenous stressor, the convergence of faulty reflexes controlled by the 5-HT (and other) systems leads to death. This hypothesis is supported by data showing medullary 5-HT abnormalities in four distinct data sets over a fourteen year period (14–17). Serotonergic abnormalities are shown in 4 additional datasets (18–21), however, as Paine et al. highlighted in 2014, these as well as many neuropathological studies of SIDS use inconsistent definitions or do not use age- and sex-matched controls which vastly limits comparison (13).

Although the 5-HT system is incredibly diverse and spans the central nervous system, the medullary portion is predominantly composed of *Pet1*-expressing neurons which regulate breathing in neonates (22). These neurons regulate the apnea-induced autoresuscitation reflex and the laryngeal chemoreflex in response to obstructive fluids in the airway, both when impaired are hypothesized contributors of SIDS. In addition, in genetic murine models, these neurons are developing in the equivalent adjusted inter-species time frame as the critical period in which SIDS is most likely to occur in humans (2–4 months of age in *Homo sapiens*, postnatal day 1–10 in *Mus musculus*) (22, 23). In summary, there is compelling evidence derived from descriptive human-based tissue research as well as mechanistic data in experimental animals supporting the notion that a

primary developmental neurological driver may be implicated in SIDS.

Nevertheless, there are also significant indications of a health disparities component to SIDS etiology. Thirty years ago the rate of SIDS in the United States (US) was considered moderate compared to other developed countries, however the US now has among the highest SIDS and postnatal mortality rates in recent years (24). SIDS, accidental suffocation/strangulation, and other ill-defined or unspecified causes of mortality are three causes of death that fall under the definition of sudden unexpected infant death (SUID). Substantial disparities are evident when comparing the rate of SIDS and SUID amongst distinct racial and ethnic groups in the US (4, 25). For instance, in 2017, 35.4 deaths per 100,000 live births occurred in the US attributed to SIDS[1]. In Native American and American Indian populations, the SIDS rate from 2014 to 2017, was 2.5 times higher than the rate of non-Hispanic white infants (95.59 and 37.65 per 100,000 live births, respectively)[1]. In addition, non-Hispanic Black infants have almost 2 times the rate of SIDS compared to non-Hispanic white infants (73.6 and 37.65 per 100,000 live births, respectively). The extent to which these distinct rates result from intrinsic genetic polymorphisms vs. other social or geographical determinants of health is highly debated in the neuropathology literature (6). It is well-known that discrete epidemiological risk factors exist for these different racial groups in regards to modifiable and non-modifiable behaviors, such as maternal smoking, breastfeeding, prematurity, and infant sleep environment (26, 27). For instance, the Aberdeen Area Infant Mortality Study, which enrolled patients for prospective observation from 1992 to 1996 revealed the following risks (in order of importance): 1st trimester maternal binge drinking, periconceptual maternal drinking, and over-bundling the infant (28). In addition to these risk factors, these decedents were also found to have abnormalities in the 5-HT system (20).

Due to these divergent perspectives, some investigators have advocated for a Triple Risk Hypothesis for SIDS. The Triple Risk Hypothesis posits that a vulnerable infant with an underlying intrinsic risk undergoes exposure to an unsurmountable exogenous stressor resulting in death (29). These intrinsic risks include genetic components such as male sex or potential polymorphisms in the serotonergic pathway, developmental risks such as prematurity, and environmental risks such as perinatal and postnatal exposure to smoking, ethanol, or other drugs (30). Extrinsic risks are physical stressors such as bed sharing, soft bedding, sleeping in non-supine positions, over-bundling, and all other risks that may lead to asphyxia or compromise homeostatic regulation. In summary, each decedent's death is due to an interaction of stressors originating in each of the "Risk Spheres" of this model. A corollary of the Triple Risk Hypothesis is that each death may have different contributions from each risk sphere, such that in some infants death follows mainly intrinsic etiologies, whereas in other infants death would result from extrinsic factors, and so forth.

Although the Triple Risk Hypothesis has represented a useful framework to understand SIDS, the inherent variabilities and confounding factors in studying human tissue make it impossible to predict which individual infant is at clear risk of sudden death. This challenge prevents delineation of clear mechanistic pathways that could be amenable to pharmaceutical intervention. Amongst these hindrances includes an inability to objectively determine the underlying etiology and relative contribution of various extrinsic and intrinsic risks of each individual decedent. Therefore, we sought to identify unique clusters of SIDS decedents in whom different risk factors and characteristics predominate. To achieve this, we obtained data from the United States National Center of Health Statistics (NCHS) managed by the Centers for Disease Control and Prevention and applied modern unsupervised machine learning models.

Neuropathology has evolved into a "big data" field with routine incorporation of machine learning and artificial intelligence informatics that spans a much larger scope than interpretation of histopathological slides. We performed an unsupervised machine learning dimensionality reduction technique known as t-distributed stochastic neighbor embedding (t-SNE). Due to the data recording methods obtained from the NCHS infant mortality files, this method was best suited for our analysis. t-SNE is a high-dimensionality reduction technique capable of modeling non-numerical data in the form of factors (31). Our analysis was narrowed to 16 categorical variables in the t-SNE computation, which permitted us to identify unique clusters of patients which were confirmed by distance statistics. The identification of patients within a heterogenous disease was initially implemented in diagnostic neuropathology in the early 2000s (32–34). This approach has been instructive in neuropathology identifying unique patient subtypes and we utilized similar methods to identify unique clusters of SIDS decedents. By identifying discrete clusters within the t-SNE analysis, we further investigated differences between these groups in terms of post-conceptional age at death and how they have been influenced over time. Lastly, our analysis sought to analyze geographic trends over time between regions and the rates of SIDS between two birth weight groups, and to compare outcomes in the US to other countries with similar or less cultural heterogeneity.

# MATERIALS AND METHODS

## Data Acquisition and Standardization

Analyses were primarily conducted using the US 1990–1991 and 1995–2017 Period Linked Birth/Death Data Sets by the National Center for Health Statistics (data files were not produced during 1992–1994)[2]. To identify SIDS decedents, we used the *International Classification of Diseases, Ninth Revision* (ICD-9) code for deaths occurring before 1999 (code 798.0) and

[1]Centers for Disease Control and Prevention, Data and Statistics for SIDS and SUID.

[2]Centers for Disease Control and Prevention. National Center for Health Statistics, Public Use Data File Documentation:1990–2017 Period Linked Birth/Death Data Sets, Hyattsville, MD: Department of Health and Human Services, 1990–2017.

*International Classification of Diseases, Tenth Revision* (ICD-10) for deaths occurring in 1999 and after (code R95) (35). These data were used to create t-SNE plots and calculate the rates of SIDS based on different variables.

In addition to analyzing US SIDS rates, our study also included calculating the SIDS mortality rates in New Zealand and Argentina. Publicly available New Zealand SIDS mortality data was obtained from the Ministry of Health from 2000 to 2016, and República de Argentina data was obtained from Dirección de Estadísticas e información en salud del ministerio de salud de la nación (Argentine Government) for 1997–2015. The US and Argentina defined SIDS occurring from birth to 1 year of age and New Zealand classified it from birth to <1 year of age in their reporting statistics (24). All countries utilized the same ICD-10 coding standard. Utilizing this metric as an international standard of comparison in SIDS rate has been previously presented by Hauck and Tanabe (24). Argentina, New Zealand and the US yields similar distribution of R95 diagnoses allowing us to trust the validity of this measure (36, 37).

SIDS mortality rates by birth weight were calculated using the total number of SIDS decedents in that birth weight class divided by the number of live infants born in the same birth weight class multiplied by 1,000. SIDS decedents with unknown birth weights were excluded from the rate calculation in analysis of all countries (0.088% excluded from US data, 4.4% excluded from New Zealand Data, 52.5% excluded from República de Argentina data).

For the US data, individual state death rate calculations were derived by dividing SIDS occurrences in a birth weight category by the number of live born infants. To avoid disclosing identifiable events, rates underwent center-scaling at zero with a standard deviation of 1 in each birth weight category. Restricted US Period Linked Birth/Death Data from 2005 to 2006 and 2014–2017 omitted state of death occurrence and were excluded from our analysis.

In order to calculate the adjusted age at death, we used the gestational age recorded in the birth certificate computed using the date of birth of the infant and last menstrual period or the clinical estimate of gestation. We considered 39 weeks gestation or higher as full term as recommended by Spong et al. (38). Gestational age (in weeks) was subtracted from 39, multiplied by 7, and subtracted from the age at death (in days) recorded on the death certificate ($n$ = 29,651; 48.51%). Infants with 39 weeks gestational age or higher did not have their age at death modified ($n$ = 30,882; 50.53%), nor did the infants who had "unknown" or "not recorded" listed as the gestational age on their birth certificate ($n$ = 585, 0.96%).

Mother's education was also recoded due to variations in the reporting codes between the unrevised and revised birth certificates. See **Supplementary Table 1** for recoding parameters.

## T-SNE Modeling and Cluster Validation

Traditional methods of studying the epidemiology of SIDS typically focus on the relationship of the event and possible risk factors associated with SIDS and each other (4, 27). To evaluate all variables, we used t-SNE, a high dimensionality reduction technique to visually observe non-linear trends in the infants dying of SIDS (39). Data are put into a model and an individual circle represents a single decedent of SIDS in the US from 1990 to 2017 (excluding 1992–1994). Variables included in our model are listed in **Supplementary Table 2**. t-SNE plots were pseudo-colored for each variable to identify clustering trends. The x and y axis represent the first and second dimension of the t-SNE analysis and similar data points are plotted near one another. Additional parameter t-SNE plots with varying perplexity and maximum iterations are included in **Supplementary Figure 1**.

In addition, we performed internal clustering validation to compute the distance between objects and their respective clusters. We started by creating a dummy variable matrix for every column to separate our factors (40). This resulted in a large dataframe (dimensions 61,118 × 128) which was challenging to interrogate with the dist() function in R. We therefore performed a permutation analysis to implement the cluster.stats() function call in R and evaluated the average distance between clusters, the average distance within clusters, and the within cluster sum of squares using the cluster.stats() function call in R. **Supplementary Figure 2** discusses our workflow for this analysis. This permutation was repeated 1,000 times, which permits identifying significance scores of up to $10^{-2}$ (represented as $10^{-2}$ due to being an estimate obtained using random sampled permutations) (41).

# RESULTS

## Epidemiological Data From SIDS Decedents Cluster Into Three Groups

By far, the most extensive dataset available for the study of SIDS in the US represents the vital statistics available through the NCHS. We therefore obtained and standardized the Period Linked Birth/Infant Death data to allow for robust analysis of SIDS decedents. These data represented 61,118 instances of SIDS decedents linked to information on both the birth certificate and death certificate, including mother's marital status, education, race, and prenatal care in addition to the infant's length of gestation, birth weight, and age at death. These parameters were chosen because variables including father's education, age, and race were inconsistently reported across the various states, alcohol usage was not collected over the entire timeframe studied, and infants with multiple variables as "unknown" clustered near one another. Clusters based on unreliable reporting led us to exclude these variables. We first tested the hypothesis that unique clusters of SIDS decedents existed by performing t-SNE analysis. Following the creation of the t-SNE plot, we pseudo-colored each circle (where each circle represents one instance of a SIDS decedent). By interacting with the data in this way, we identified trends in the data as to which variable created the most segmented groups. Birth weight was the most significant driver separating the groups (**Figure 1A**). Infants with very low birth weight (indigo) and low birth weight (light blue) clustered closer together compared to normal birth weight infants (pink), with very little intermingling between groups at the border (**Figure 1A**). In summary, even when using multiple parameters in the t-SNE function call, birth weight was the most significant driver in group separation (**Supplementary Figure 1**).

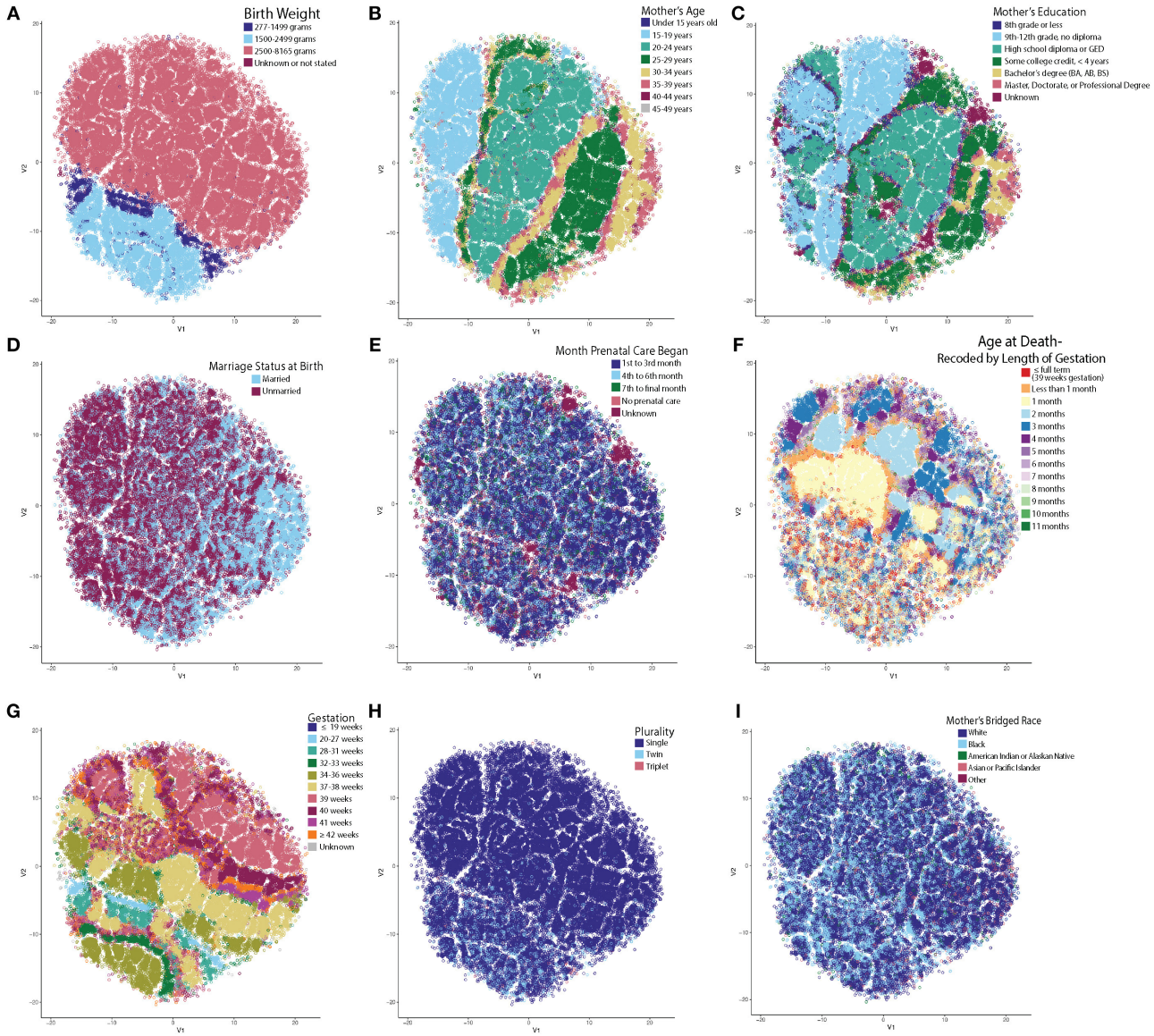


**FIGURE 1 |** t-SNE Reveals Unsupervised Clustering Patterns. Each circle represents one SIDS decedent and has been pseudo-colored for each variable to identify clustering trends. X and Y axes represent the first and second dimension of the t-SNE analysis and units are arbitrary. **(A)** Infant's birth weight created the most distinct separation of clusters. Very low and low birth weight infants clustered together with very little intermingling between the normal birth weight infants. **(B)** Mother's age at time of birth. **(C)** Mother's highest attained education at time of birth. Note that on the left in B and C, are teenage mothers still in high school compared to the right side of the plot with mothers who are older and have a higher education. **(D)** Mother's marital status at birth. Note that the older moms (30+) are more frequently married. **(E)** Month prenatal care was initiated. **(F)** Age at death recoded by length of gestation. Note that the low birth weight infants and infants who are born to older, more educated, and married mothers have more heterogenous clustering patterns. **(G)** Low birth weight infants dying from SIDS have shorter lengths of gestation and less frequently reach full term gestation (39+ weeks). **(H)** Multiparous pregnancy. Infants in the low birth weight cluster have a higher percentage of twin and triplet occurrences compared to single births. **(I)** Mother's bridged race. It is known that there are large racial disparities in the rate of SIDS. Note the high density of Black mothers in the low birth weight cluster and how there is a larger percentage of mothers who are white in the cluster forming on the right half (identified as group 2 in **Figure 2**).

Upon further data exploration, we identified a smaller, second group within the normal birth weight cluster (**Figures 1B–E**). Although not as easily segregated visually in the t-SNE as the birth weight clusters, we noticed that the t-SNE algorithm clustered decedents whose mothers were 25 years of age or older (**Figure 1B**), attended college for at least 4 years (or completed a bachelor's degree or higher) (**Figure 1C**), were married (**Figure 1D**), and began prenatal care in the first trimester (**Figure 1E**). Based on known demographic trends, these decedents likely came from families that had more stable financial and home environments. We also noted that a third group, located in the center of the t-SNE

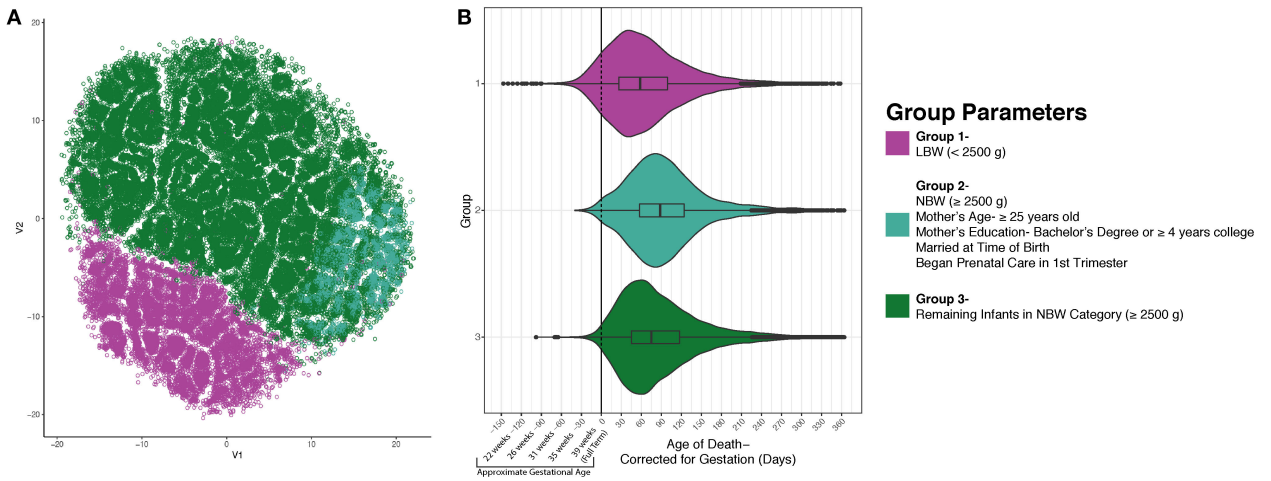


**FIGURE 2 |** SIDS Decedents Cluster into Three Distinct Groups. After using unsupervised modeling with t-SNE, we identified 3 discrete groups based on infant birth and death data as well as maternal characteristics **(A)**. Group 1 (magenta) is formed by the infants who weighed <2,500 grams at birth. Group 2 is less segregated and found plotted on the right side in teal. This group is comprised of decedents who are normal birth weight and had mothers who are 25 years of age or older, had a bachelor's degree or ≥4 years of college, married, and began prenatal care in the first trimester. Group 3 (green) are the remaining infants in the normal birth weight category. **(B)** After identifying three discrete groups, we sought to identify differences in postconceptional age at death. There is a statistically significant distinction in the average age at death for each group ($p < 2e^{-16}$). Nearly 10% of the infants in group 1 passed away before reaching full term gestation which could indicate a different etiology of SIDS.

plot, showed significant clustering by the adjusted age at death (**Figure 1F**).

In these three discrete groups, we noticed age at death (**Figure 1F**) did not show significant clustering within the low birth weight group (group 1) or in group 2 comprised of older, more educated, and married mothers who started prenatal care in the first trimester. In addition, the infants in group 1 had a different pattern of clustering by gestation (**Figure 1G**) and a larger percentage of infants in this group were from multiparous gestations (**Figure 1H**). Based on a chi-square test, there was a statistically significant higher percent of white mothers in group 2 than groups 1 and 3 (**Figure 1I**). We further evaluated the distance between clusters (**Supplementary Figure 2**), and found that the mean within cluster sum of squares of these three clusters was 45,231 ($SD = 82.9$, $p < 10^{-2}$), the mean average within cluster distance was 4.23 ($SD = 0.0039$, $p < 10^{-2}$), and the mean average between cluster distance was 4.48 ($SD = 0.0046$, $p < 10^{-2}$). We conclude that three discrete groups of decedents were identified by machine learning-based evaluation of the NCHS epidemiological data.

Having identified these three groups using an unsupervised learning approach, we next sought to identify potential differences between these groups that might provide insight into the etiology of SIDS. In **Figure 2A**, group 1 (low birth weight) is illustrated in magenta ($n = 12{,}310$), group 2 (normal birth weight decedents with mothers attaining higher education, 25 years or older, married, and in whom prenatal care commenced in the first trimester) in teal ($n = 5{,}143$), and group three (remainder of decedents in the normal birth weight category) in green ($n = 43{,}665$). Following the identification of these three groups, we next tested if the mean age of death was distinct between groups. When comparing the three groups' postnatal age at death based on the death certificate, we noticed that each group had similar peak ages at death. However, since low birth weight is highly related to prematurity, we recalculated each decedent's postconceptional age so that we could plot all decedents along the same timeline. When plotting the three distinct groups based on their postnatal age corrected for gestation, we discovered differences in the distribution and arithmetic mean of age at death between the three groups. In group 1, the mean age at death was 67.89 days ($SD = 61.95$); group 2's mean age at death was 96.80 days (SD = 60.71); and group 3's mean age at death was 88.22 days ($SD = 62.82$). Evaluation of statistical significance by ANOVA/TukeyHSD test demonstrated a significance of $p < 2e^{-16}$ and an adjusted $p$-value of $2e^{-16}$, $2e^{-16}$, and $3e^{-14}$, for groups 2-1, groups 3-1, and groups 3-2, respectively. These data indicate that the mean peak incidence of SIDS within these three groups occurs at distinct neurodevelopmental ages. In addition to the groups having significantly different postconceptional ages at death, it is important to note that many infants (∼10%) born prematurely in group 1 died of SIDS before they reached full-term gestation with a calculated $p$-value $<2e^{-16}$ using a chi-square test. This larger percentage of infants dying before reaching full term gestation in the low birth weight group could suggest that in this group of SIDS decedents, neurodevelopmental pathologies may play more important roles in SIDS pathogenesis relative to the other groups.

## Geographical Discrepancies of SIDS Mirrors Drivers of Health Disparity in the US

After determining there were three discrete groups based on maternal and infantile characteristics, we wanted to see how each

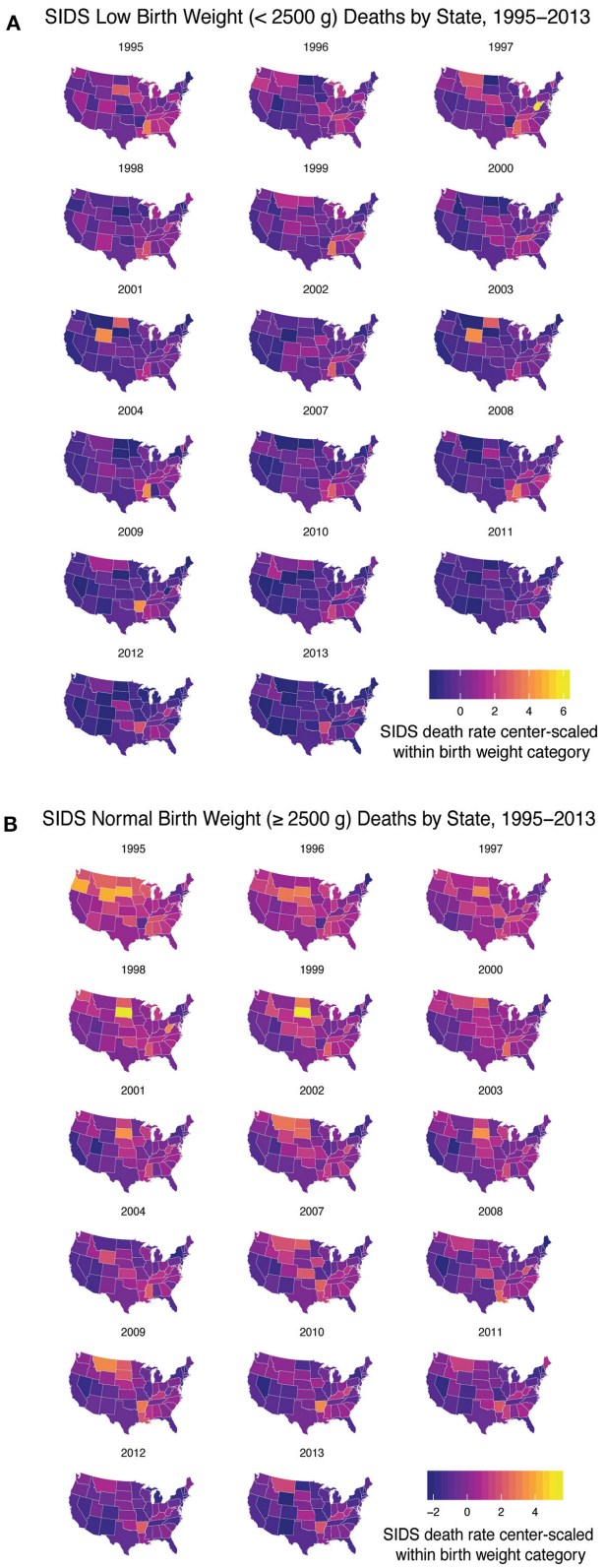


**FIGURE 3 |** SIDS Rates Disparities Persist Across the US. From 1995 to 2013 (excluding 2005–2006) we analyzed the geographical heterogeneity of SIDS *(Continued)*

**FIGURE 3 |** rates based on decedent birth weight. **(A)** Low birth weight infants had a higher rate of SIDS in West Virginia, Arkansas, Wyoming, Mississippi, North and South Dakota, Montana, and Louisiana and the District of Columbia. **(B)** Normal birth weight decedents had higher rates of SIDS mirroring the states that had high rates of low birth weight SIDS. Geographical disparities persisted over time based on infant birth weight.

state performed over time in regards to reducing the rate of SIDS in these groups. Due to limited data availability, we were only able to perform this analysis with group 1 (infants weighing <2,500 g) and a second group based on infants born weighing 2,500 g or greater. Data were center-scaled within birth weight categories including geographical information on SIDS death from 1995 to 2013 (excluding 2005–2006). We found over time, states have generally reduced the rate of SIDS for these two groups, however, disparities are still present. In the low birth weight group, states that had higher rates of SIDS include West Virginia, Arkansas, Wyoming, Mississippi, North and South Dakota, Montana, Louisiana, and the District of Columbia (**Figure 3A**). In the normal birth weight group, states that had higher rates of SIDS mirrored many of the low birth weight SIDS rates and include South and North Dakota, Wyoming, Oregon, Arkansas, West Virginia, Montana, Mississippi, and Louisiana (**Figure 3B**). In general, the rates were highest in the Southeast and Northwest regions and lower in the Pacific and Northern Atlantic coastal regions of the US. We also note that high correlation between low birth weight SIDS rate and normal birth weight SIDS rate by state geography ($p < 0.001$), indicating that a strong geographical heterogeneity in SIDS incidence across the US. We also note that regions of SIDS hotspots mirror the geographic distributions of African American and Native American populations as well as regions with lowest GDP per capita. This correlation was determined in two separate linear regression models analyzing the 2013 SIDS rate based on race (i.e., response variable) in each state compared to the GDP (i.e., predictor variable). State GDP shows a significant linear relationship with the rate of white infants dying of SIDS but no correlation with Black infants. The linear regression data are reported in **Supplementary Table 3**.

## Implementation of Clinical Guidelines Shows Distinct Impact in Decreasing SIDS Rates in Different SIDS Clusters

To evaluate the progress of SIDS reduction over time, we plotted the rate of SIDS within the two distinct clusters based on birth weight from 1995 to 2017. Clinical guidelines, such as the Back to Sleep campaign, had a momentous effect on declining the SIDS rates in the US beginning in 1994, but the SIDS rate plateaued in the early 2000s (25). However, when calculating by birth weight, the rate of SIDS in infants <2,500 g has steadily decreased since 2008 (**Figure 4A1**—magenta). This may be due partly to the American Academy of Pediatrics' (AAP) safe sleep guideline describing the risks involved with bed-sharing. However, there is a significantly larger number of infants in the normal birth weight group (seen in **Figure 4A2**—green) that

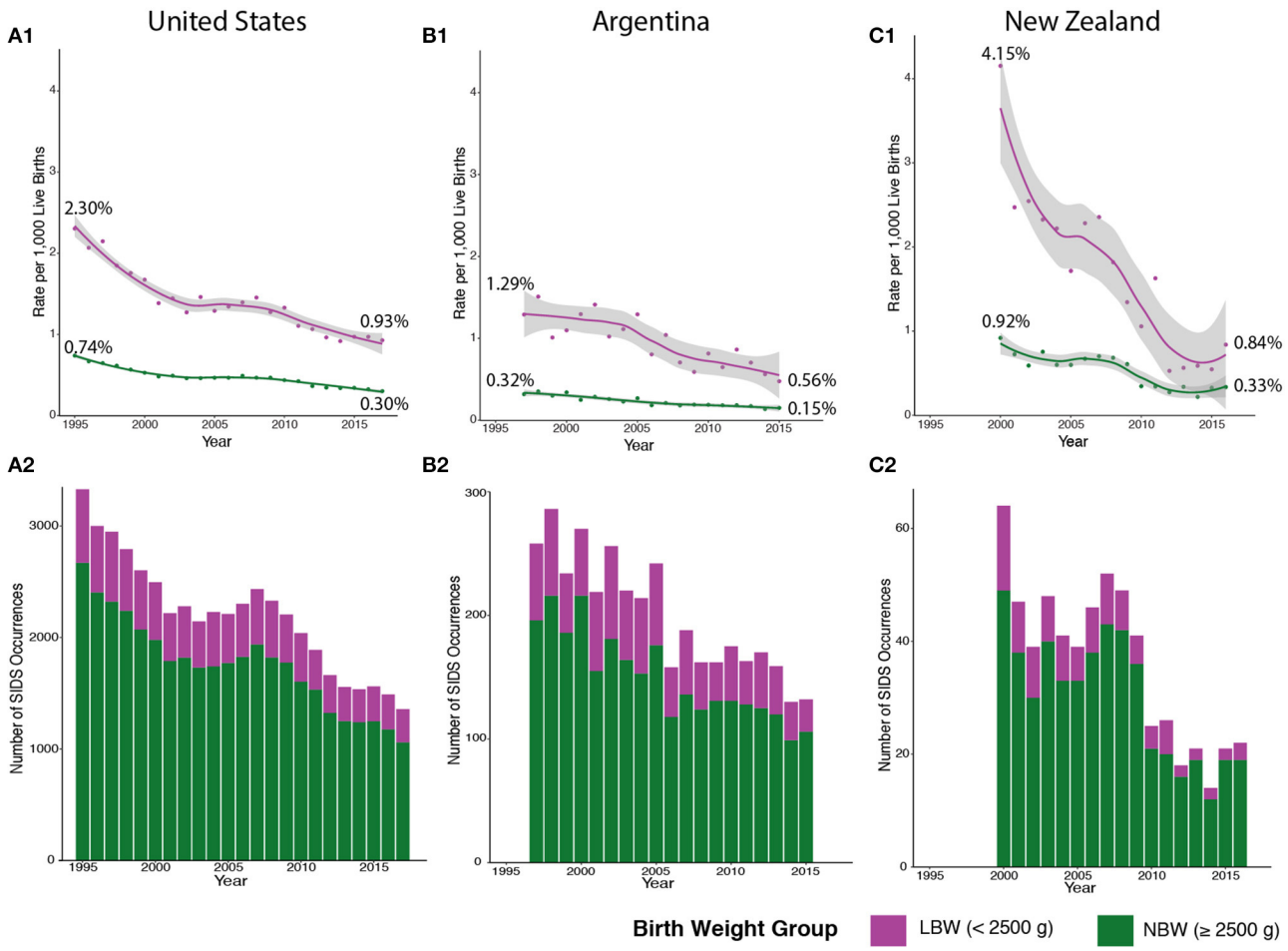


**FIGURE 4 |** SIDS Rates in the US, Argentina, and New Zealand Follow Similar Trends. We broadened our analysis to include countries with different levels of cultural heterogeneity compared to the US. SIDS rates in low birth weight (LBW) infants (colored in magenta) are higher in the US **(A1)**, Argentina **(B1)**, and New Zealand **(C1)** compared to normal birth weight (NBW) infants (colored in green). There is also a more drastic decline in LBW SIDS rates compared to a stable SIDS rate in NBW infants. However, fewer cases of SIDS are comprised of LBW infants in the US **(A2)**, Argentina **(B2)**, and New Zealand **(C2)**. Major reductions in SIDS mortality are not seen in NBW infants in the US (1995–2017), Argentina (1997–2015), and New Zealand (2000–2016).

are not being equally impacted by implementing these clinical guidelines (**Figure 4A1**—green). Although the AAP's clinical guidelines are important, these discrete groups of SIDS decedents are not experiencing as rapid a reduction in incidence compared to the low birth weight group.

We broadened our analysis to see if the two distinct groups we identified had similar trends in other countries with different levels of cultural heterogeneity than the US (GDP per capita of $62,794.59) (42, 43). In both Argentina [lower cultural diversity compared to US with a GDP per capita of $11,683.95 (USD)] and New Zealand [similar cultural diversity to US and a GDP per capita of $41,945 (USD)], lower birth weight infants also have a higher rate of SIDS mortality compared to normal birth weight infants (**Figures 4B1,C1**—magenta). These data indicate that across three countries with different levels of cultural heterogeneity and GDP, similar trends in the rates of SIDS can be identified. In addition, the lower birth weight SIDS decedents are a much smaller number compared to normal birth weight SIDS decedents in all the countries (**Figures 4B2,C2**—green), and the rate at which this subgroup undergoes reduction in incidence rates over time has similarly been more rapid compared to the normal birth weight group. Note that New Zealand (**Figure 4C**) has been very successful in reducing mortality in the low birth weight group, with recent data from 2016, demonstrating complete overlap in the 95% confidence intervals of the fitted loess models of the normal birth weight and low birth weight data. Furthermore, the New Zealand low birth weight incidence has stabilized at ∼0.6% mortality from 2012 to 2015, potentially indicating that the low birth weight trends may converge to the normal birth weight group. In summary, we conclude that major drivers in reduction of SIDS occurrences include reduction in mortality in low birth weight infants. These data suggest that additional insights into the developmental neuropathology of SIDS may be required to identify molecular pathways that could be targeted to prevent SIDS incidents.

# DISCUSSION

## Autonomic Control and Postnatal Breathing Disorders

Autonomic control of breathing is impaired in human pediatric breathing disorders such as apnea of prematurity (AOP) (44, 45), Rapid Onset Hypoventilation Hypothalamic and Autonomic Dysfunction (ROHHAD) syndrome (46, 47), Rett syndrome (48), and Congenital Central Hypoventilation Syndrome (CCHS) (49). The full-term human brain doubles in size, reaching 75% of the adult brain weight by the end of the first postnatal year (50), and it is unclear how brainstem circuitry changes during this critical time period. Although anatomical deficiencies have been noted in the SIDS brainstem [such as arcuate nucleus hypoplasia (12)], it is unclear how other human brainstem structures function in homeostasis and if they are dysfunctional in SIDS. In this context, the study of known pediatric breathing disorders is particularly instructive. CCHS is a genetic pediatric breathing disorder caused by mutation in the gene *Phox2b*. Severe impairment in autonomic nervous system breathing control without cardiorespiratory disease is characteristic of CCHS (51, 52). Lack of $CO_2$ chemosensitivity leads to diminutive tidal volumes and monotonous respiratory rates, resulting in rising blood $pCO_2$ levels. Thus, the pathophysiology of CCHS may be instructive to understanding central $pH/pCO_2$ chemosensation, and insights derived from CCHS are applicable to other pediatric breathing disorders. Human patients who die of CCHS in the perinatal period show neuronal loss of locus ceruleus as a common neuropathological finding (53, 54). Mouse studies have demonstrated that in severe forms of CCHS, developmental loss of visceral motor neurons result in non-cell autonomous deficiencies occurring in known brainstem respiratory pattern generators, including the $CO_2$ chemosensitive retrotrapezoid nucleus and the preBötzinger complex, the main neonatal respiratory central pattern generator (55). Although such studies in rodents have been instructive in elucidating autonomic circuits, to date the study of rare genetic disorders such as CCHS and Rett syndrome has not resulted in knowledge that is generalizable to elucidating the molecular underpinnings of SIDS.

## Potential Neurodevelopmental Mechanisms Underlying SIDS

The intersection of neural, respiratory, and cardiovascular responses is at the forefront of SIDS mechanistic hypotheses. These hypotheses predict that the cause of death would be due to abnormal generation of respiration and preceded by multiple failures of protective mechanisms in the putative respiratory pathway (30, 56). These failures include a life-threatening event such as hypercapnia, hypoxia, asphyxiation in bedding, etc., and the failure of head lifting or turning. Some of these deficits are due to well-known paradoxical physiological responses in newborn mammals. For instance, in all newborn mammals, acute hypoxia results in a rapid rise in ventilation driven mainly from carotid bodies (Phase I) followed by a CNS-mediated repression of the ventilation (Phase II/Roll-off) which overshoots baseline and can even be elicited using brainstem slices (57). Recent studies in mice have shown that exposing newborn mouse pups to complete anoxia results in reduction in heart rate and respiration and does not recover correctly when *Pet1*-derived neurons, which generate a large population of 5HT neurons, undergo silencing (58). Along these lines, an infant could undergo failure of arousal, hypoxic coma, bradycardia and gasping, and failure of autoresuscitation (30). Emergency brainstem reflexes should trigger a rescue response to reinstate eupnea, however, certain disease states could impair this response, resulting in death (59). In premature infants, episodes of apnea are prevalent and separated into two categories: awake apnea and sleep apneas. SIDS decedents are often found following a period of sleep; however, it is still unknown whether death occurred during the sleep phase or following brief arousal.

The most common cause of sleep apneas in premature infants are respiratory pauses associated with the laryngeal chemoreflex (LCR). The LCR is characterized by a preceding period of hypoxia with bradycardia, swallowing, and a cough to clear the airway of obstructive fluids. Discovered in piglets, the swallowing and coughing response during the LCR exhibits low complexity values, signifying it is induced by a homogenous group of neurons (60). Piglets early in age also exhibited shorter duration of respiratory activities, suspected to be due to the rapid development of this neuronal system within the first month of life (60). In the event that a vulnerable infant has an apneic occurrence and they have susceptibility in the homogenous neuronal population controlling for the LCR, this puts the infant in a position they cannot survive (60).

In addition to the rapid development of the neurons controlling the LCR postnatally, the majority of postnatal proliferating cells in the pons are oligodendrocytes (61, 62). Prenatal insults might impact the differentiation of oligodendrocytes, leading to the failure of myelination of important pathways spanning the length of the brainstem. An extensively studied pathway in regards to SIDS is the serotonergic (5-HT) pathway. Effector nuclei responsible for cardiorespiratory integration, pharyngeal and laryngeal airway control, respiratory rhythm generation, and parasympathetic and sympathetic innervation may be impacted by the lack of myelination. In addition, whether the 5-HT abnormalities are causative and have pathological significance or correlative and a genetic predisposition in this pathway in a subset of SIDS decedents is yet to be determined (63).

## Recommendations for Forensic Neuropathology

In the US, sudden infant death cases fall under the jurisdiction of the local medicolegal investigation systems, which are currently a mixture of coroner, medical examiner, and hybrid systems. This heterogeneity of systems and investigative practices combined with funding limitations and a national shortage of forensic pathologists has led to inconsistencies in the certification of sudden, unexpected deaths in infants (64, 65). In addition, there has been increasing reluctance by forensic pathologists to certify a death as SIDS or SUID, partly due to some forensic pathologists

viewing SIDS is a diagnosis of exclusion, making it difficult to diagnose if there are any limitations in the scene investigation, lack of clear circumstances around the death, or if external risk factors, such as unsafe sleep conditions, are present. As a result, there has been a shift toward using "Undetermined" as the cause of death in many of these cases (65, 66). Unfortunately, these variations in cause of death classification directly impact the ICD-10 codes used by epidemiologists and researchers, as SIDS is given an R95 code and "Undetermined" is given an R99 code.

In 2019, the National Association of Medical Examiners' (NAME) Panel on Sudden Unexpected Death in Pediatrics, an expert panel composed of medical examiners, pediatricians, and federal agency representatives, released "Unexpected Pediatric Deaths: Investigation, Certification, and Family Needs" which recognized this issue and the need for consensus guidelines for the investigation and certification of unexpected pediatric deaths (67). As such, they put forth recommendations for a standardized system of completing death certificates for these cases. Sudden, unexpected infant deaths that remain unexplained after a thorough investigation and autopsy were recommended to be certified as "Unexplained Sudden Death" as the cause of death (thereby generating an R95 ICD-10 code) with an additional indication of whether intrinsic factors (defined as natural conditions or risk factors associated with abnormal physiology) or extrinsic factors (defined as conditions in the infant's immediate environment that are a potential threat to life but cannot be deemed the cause of death with reasonable certainty) were present. The specific intrinsic and/or extrinsic factors are not entered on the death certificate, but rather the NAME Panel proposed they be incorporated into a synoptic report that would become a component of the autopsy report. Synoptic reports are commonly used in surgical pathology reports for cancer diagnoses and have allowed for increased consistency across pathologists and institutions, however synoptic reporting has not been widely used in the forensic setting. Recognizing the desire to capture the complex information regarding unexpected pediatric deaths, the NAME Panel's recommended synoptic report includes information regarding the extent of the investigation, medical history of the infant, specifics of the autopsy procedures performed, toxicology, ancillary studies, and radiologic studies. Thus, not only is the synoptic report a useful resource for capturing data but it also highlights any limitations of the investigation or autopsy so that these factors can be considered in data analyses. Furthermore, they had specific recommendations for when to use "Undetermined" as the cause of death. "Undetermined (Not otherwise specified)" should be used for deaths where the cause of death cannot be determined due to circumstances or findings that may raise uncertainty about the manner of death, those that were not considered to be sudden deaths, or those with competing causes of death. Cases where the cause of death could not be determined because of substantial limitations in the investigation or autopsy examination were recommended to be certified as "Undetermined (Insufficient Data)." While this classification system would still not capture all the potential SIDS cases (particularly in systems where resources and funding are limited for investigations and autopsies), it does provide more guidance to forensic pathologists that would hopefully allow for more consistency in reporting.

## DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: privacy or ethical restrictions imposed by the United States Centers for Disease Control. Requests to access these datasets should be directed to nvssrestricteddata@cdc.gov.

## AUTHOR CONTRIBUTIONS

JB conceptualized and designed the study, carried out analysis, and drafted the initial manuscript. VC obtained Argentine infant mortality data, reviewed, and revised the manuscript. JS and JZ refined the analysis, reviewed, and revised the manuscript. AS and CP critically reviewed and revised the manuscript. JO conceptualized the study, refined analysis, reviewed, and revised the manuscript. All authors approve the final manuscript as submitted.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2020.594550/full#supplementary-material

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# Elevated Neurokinin-1 Receptor Expression in Uterine Products of Conception Is Associated With First Trimester Miscarriages

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**Background:** Miscarriage is a common complication of early pregnancy, mostly occurring in the first trimester. However, the etiological factors and prognostic and diagnostic biomarkers are not well known. Neurokinin-1 receptor (NK-1R) is a receptor of tachykinin peptide substance P (SP) and has a role in various pathological conditions, cancers, but its association with miscarriages and significance as a clinicopathological parameter are not studied. Accordingly, the present study aimed to clarify the localization and expression for NK-1R in human retained products of conception (POC). The role of NK-1R is not known in miscarriages.

**Materials and Methods:** NK-1R expression was assessed in POC and normal placental tissues by immunohistochemistry. Three- to four-micrometer-thin sections of formalin-fixed paraffin-embedded tissues were used for this purpose. Tissues were processed and then immunohistochemically stained with NK-1R antibody. Brain tissue was used as control for antibody. Protein expression was evaluated using the nuclear labeling index (%). Tissues were counterstained with 3,3′-diaminobenzidine (DAB), and microscopy was performed at 10× , 20× , and 40× magnifications.

**Results:** Ten human POC tissues and 10 normal placental tissues were studied by immunohistochemistry to demonstrate the localization of NK-1R. The expression of NK-1R protein was high in all the cases of both groups. NK-1R expression showed no notable differences among different cases of miscarriages as well as normal deliveries at full term regardless of the mother's age and gestational age at which the event occurred. Statistically, no difference was found in both groups, which is in agreement with our hypothesis and previous findings.

**Conclusion:** The expression of NK-1R was similar in all the cases, and it was intense. It shows that dysregulation of NK-1R along with its ligand SP might be involved in

miscarriages and also involved in normal delivery. Our results provide fundamental data regarding this anti-NK-1R strategy. Thus, the present study recommends that SP/NK-1R system might, therefore, be considered as an emerging and promising diagnostic and therapeutic strategy against miscarriages. Hence, we report for the first time the expression and localization of NK-1R in POC. We suggest NK-1R antagonist in addition to the immunoglobulins and human chorionic gonadotropin to diagnose and treat spontaneous miscarriages.

**Keywords: miscarriages, abortions, neurokinin 1 receptor, substance P, NK-1R antagonists**

## INTRODUCTION

Miscarriages or spontaneous abortion in the initial stage of pregnancy is a common problem in the first trimester of pregnancy (Strommen et al., 2017). Many factors are involved in it, and it is a complex phenomenon [27]. Approximately 15% of pregnant females miscarry spontaneously without any known cause (Shetty and Narasimha, 2016). Miscarriages are further divided into incomplete, complete, missed, and anembryonic miscarriages (Kling et al., 2018). Fifty percent of causes are still unknown, and no indicators have been identified in them. Tests to diagnose these cases include an ultrasound scanning and measurement of human chorionic gonadotropin (hCG). In this way, the patients at risk are identified. However, there is a dire need to explore potentially efficient biomarkers for the diagnosis of at-risk patients earlier than the onset of clinical symptoms or the unfortunate occurrence of event (Monteiro et al., 2020). It will not only provide a diagnostic strategy but also pave way for therapeutic interventions to manage such cases. For this purpose, neurokinin-1 receptor (NK-1R) is explored in the current study to evaluate its expression and localization in the products of conception (POC) tissues after a miscarriage.

NK-1 is a receptor of substance P (SP) protein, which is one of the peptides released from sensory nerves, and causes the enhancement of cellular excitability in several human tumor cells (Javid et al., 2020). There are two distinct conformational isoforms of NK-1R: a full-length NK-1R (NK-1RF) isoform and a truncated NK-1R (NK-1RT) isoform. The NK-1RT isoform lacks the terminal cytoplasmic 96-aa residues (Chernova et al., 2009). Both of these isoforms have the same binding affinity for SP but different affinities for NKA. The NK-1R has a relatively long 5′ untranslated region compared to the other tachykinin (TK) receptors, which is preceded by a single TATAAA sequence (Hershey and Krause, 1990).

The neurokinins are a class of peptide signaling molecules that mediate a range of central and peripheral functions including pain processing, gastrointestinal function, and stress responses like hematopoiesis (Chow et al., 2020), wound healing (Suvas, 2017), increased vascular permeability, neurogenic inflammation, leukocyte infiltration, cell survival, and anxiety (Schank, 2020). It is also involved in carcinogenesis as reported in many studies and leads to metastasis (Munoz et al., 2014, 2015; Mehboob et al., 2015; Javid et al., 2019). SP has a variety of physiological functions in humans, particularly, nervous, immune, and cardiovascular systems (Vilisaar and Arsenescu, 2016). Mainly, SP is a brain–gut hormone, and its receptor is present in brain regions but also in peripheral tissues. SP binds to NK-1R to carry out transmission of pain, secretions from the paracrine and endocrine system, vasodilation, and proliferation of cells (Garcia-Recio and Gascón, 2015; Amiri and Hashemy, 2019). It is a neuromodulator, neurotransmitter, as well as a neurohormone.

TKs possess a widespread distribution in central and peripheral nervous system that is undoubtedly a major source of these peptides. However, TKs also have a limited distribution in non-neural structures represented by the irregular and sparse localizations in which they display known and unknown functions. In neuronal cells, the active TKs act as neurotransmitters/neuromodulators; in non-neuronal cells, as autocrine, paracrine, and endocrine regulators.

SP brings most of its cellular activities after binding to the G protein-coupled receptor (GPCR) NK-1R. NK-1R has 407 amino acids and is encoded by TACR-1 gene. It is located on the cell surface (Helke et al., 1990). SP binds to heterotrimeric GPCR, with a preference for Gs and Gq like all TK receptors. Binding of receptor to Gs stimulates adenylyl cyclase and cyclic AMP production, whereas binding of receptor to Gq stimulates the phosphatidylinositol cascade (Nakanishi, 1991). Upon binding of SP to NK-1R, a signal transduction cascade is initiated by internalization of SP–NK-1R complex (Quartara and Maggi, 1997) that activates phospholipase C (PLC).

NK-1R, stimulated directly, may cause vasodilation of feto–placental blood vessels (Lowry and Woods, 2018). In a study, SP has been found to be expressed in trophoblastic cell tissues, but not observed in blood vessels of the fetus. Messenger RNA (mRNA) of NK-1R has been reported in placental tissue (Munoz et al., 2010; Obstetrics Subgroup et al., 2016).

In our previous study, we have postulated a theory based on evidence that SP/NK-1R expression may be variable in different developmental phases of humans whereby its expression is lower in normal fetus and elevated soon after birth and in infants. However, it decreased in adults, while if it is *vice versa*, it may cause sudden unexpected deaths (Mehboob, 2017). Accordingly, the present study aimed to clarify the localization and expression of NK-1R in human retained POC. The role of NK-1R is not known in retained POC. Identification of the cause is challenging, and there are no effective measures available for treatment.

## MATERIALS AND METHODS

### Tissue Samples

The present study was approved by the Ethical Review Committee of The University of Lahore. Ten samples of POCs after different stages of spontaneous miscarriages and 10 normal placental tissues after full-term successful delivery were obtained from the Gynaecology Department, The University of Lahore Teaching Hospital, Lahore. The ages of the females ranged from 20 to 40 years with a mean age of 29 ± 11.5 years in females whose POCs were used in this study. The mean age of females who delivered successfully was 31 ± 7.5 years, and their normal placental tissue was obtained. The gestational age ranged from 1 and 12 weeks, and the mean weight of the fragments was 295 g. The mean gestational age of females with full-term normal delivery was 37 ± 1.3 weeks.

### Immunohistochemical Staining for Neurokinin-1 Receptor

Human POC tissues and normal placental tissues were studied by immunohistochemistry to demonstrate the localization of NK-1R. Tissues of POCs were fixed in 10% formalin and then embedded in paraffin. Four-micrometer-thick serial sections were cut and processed for immunohistochemical detection of NK-1R. Antigen retrieval was done in 10 mM citrate buffer (pH 6.0), followed by peroxidase blocking (Dako Cytomation A/S) on the sections, and then heated at 100°C for 60 min. NK-1R antibody (Abbott; 1:1,000) was applied and incubated for 30 min at 37°C and incubated in Universal Secondary Antibody (Roche Diagnostics KK) for 20 min at 37°C. DAB Map detection kit (Roche Diagnostics KK) was used for visualization. Nuclei were counterstained using a hematoxylin counterstain reagent (Roche Diagnostics KK).

We studied representative samples of POCs and placental tissues. The evaluation of all slides was done by two independent pathologists. In each slide, high-power microscopic fields were evaluated using a 10×, 20×, and 40× magnification. The presence or absence of staining and the intensity of the immunoreactivity were noted, as well as the number and type of cells. Intensity of staining was observed as a brown staining. The localization of staining, whether or not the staining was localized in the nucleus, cytoplasm, or plasma membrane, was also observed. The results were recorded as positive when they showed cellular and/or plasma membrane staining ranging from moderate to strong in more than 10% of the cells. By consensus among the pathologists, the intensity of the immunoreactive

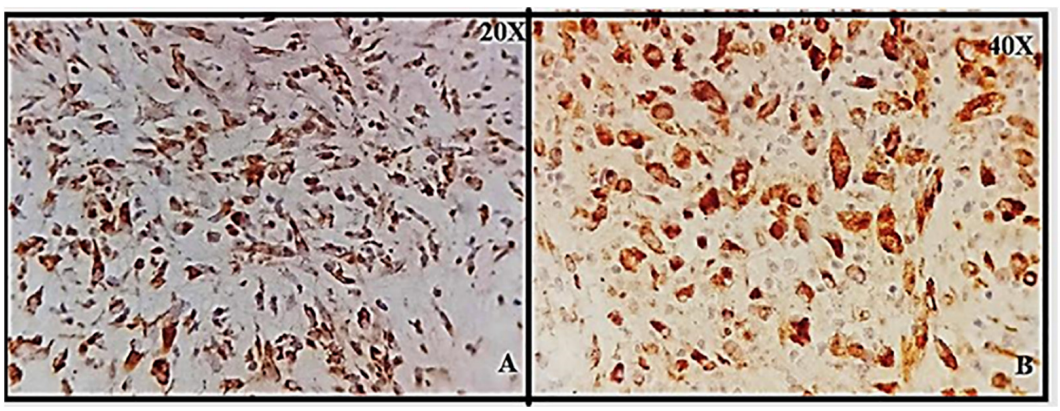


**FIGURE 1 | (A,B)** Granular cytoplasmic positive staining in brain glial cells; positive control for neurokinin-1 receptor (NK-1R) at 20 and 40×.

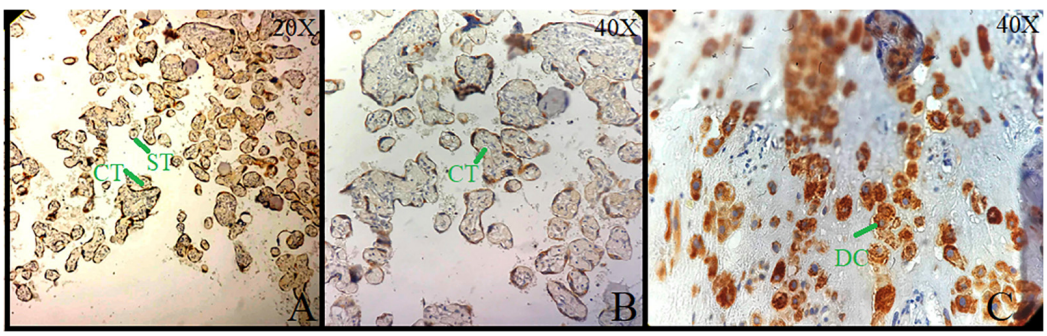


**FIGURE 2 |** Immunolocalization of neurokinin-1 receptor (NK-1R) in normal placental tissue. **(A)** Strong cytoplasmic staining in cytotrophoblast (CT), syncytiotrophoblast (ST) at 20×. **(B)** Cytoplasmic staining in CT at 40×. **(C)** Sheets of decidual cells (DCs) with positive membrane and cytoplasmic NK-1R at 40×.

cells was scored as follows: when less than 10% of the total cells were stained, the number of immunoreactive cells was considered low (+1); it was considered moderate when 10–40% were stained (+2) and high when more than 40% were stained (+3) (Shima et al., 2010). The specimens were examined and photographed at 10×, 20×, and 40× magnification utilizing a digital microscope camera (Olympus AX80 DP21; Olympus, Tokyo, Japan) interfaced with a computer. All protein levels were evaluated using the nuclear labeling index (%), recorded as the percentage of positively stained nuclei in 100 cells in the selected area.

# RESULTS

## Neurokinin-1 Receptor Protein Expression in Products of Conception and Placental Tissue Determined by Immunohistochemical Staining

Brain tissue was taken as a positive control for NK-1R, and it showed intense staining of +3 in all the cells (**Figures 1A,B**). The expression of NK-1R protein was high in all the cases of POCs when evaluated in all the stages of miscarriages. NK-1R expression showed no notable differences among different cases of miscarriages regardless of the age of females and the gestational age at which the event occurred. The NK-1R was widely distributed in the fetal membranes and placental tissues. The staining was high in epithelial cells of decidua, trophoblast of the fetal membranes, and chorionic villi (cytotrophoblast and syncytiotrophoblast). NK-1R was expressed in all the cells of POCs, whether maternal or fetal, in their epithelial membranes and nuclei. We determined the immunohistochemical staining of NK-1R, which is expressed mainly in the brain (**Figures 1A,B**). NK-1R levels were high and showed intense +3 expression in normal placental tissue (**Figures 2A–C**) as well as POCs (**Figures 3A–F**), with not much difference. Furthermore, we evaluated the nuclear labeling index of NK-1R using semi-serial sections. The NK-1R also showed no difference when comparing the cases within the POC group or normal placental group.

## Statistical Analysis

Median and interquartile range (IQR) were calculated for patient's age and gestational age. Mann–Whitney *U*-test was applied to check the significant difference between NK-1R staining and groups. $P < 0.05$ was considered significant.

**TABLE 1 |** Mann–Whitney *U*-test on neurokinin-1 receptor (NK-1R) staining and groups.

| Tissue | Female's age (years) (median + IQR) | Gestational age in weeks (median + IQR) | NK-1R staining (median + IQR) | *P*-value |
|---|---|---|---|---|
| POCs | 29 ± 11.5 | 7 ± 3.3 | 3.00 ± 0.0 | 0.7391 |
| Normal placenta | 31 ± 7.5 | 37 ± 1.3 | 3.00 ± 0.0 | |

The median age of females whose retained POC were obtained was 29 ±11.5 years, and gestational age was 7 ± 3.3 weeks. The median age of females whose normal placenta was obtained was 31 ± 7.5 years, and gestational age was 37 ± 1.3 weeks. There was insignificant association between NK-1R staining and groups (**Table 1**).

# DISCUSSION

This study reports for the first time about the detailed expression of NK-1R in POC at an early gestational age in the first trimester. Secondly, SP/NK-1 receptor system dysregulation may be involved in the pathology of pregnancy, such as abortion (Vilisaar and Arsenescu, 2016; Amiri and Hashemy, 2019), preeclampsia (Shima et al., 2010), and preterm birth. We describe here the immunolocalization of NK-1R in human POC, and we provide evidence that NK-1R is expressed in the nucleus. All these observations suggest that the NK-1R and SP have a role in the physiology of pregnancy. In our view, the demonstration that NK-1R in uterine products is associated with first trimester miscarriages has an important functional implication. NK-1R has a role in female reproduction. It has been known that mRNA for preprotachykinin-A, which encodes SP, is expressed in bovine corpus luteum (CL) of an early developmental stage; CL with a retained oocyte shows that the muscular apparatus of the preovulatory follicle has a role in oocyte expulsion and that the follicle wall contraction was missing in the mutant group. This may suggest luteinized unruptured follicle syndrome in humans (Patak et al., 2000).

There are several reports on the involvement of TKs in reproduction (Clement et al., 2009). All the TKs and their receptors are found to be expressed in the uterus of super-ovulated and unfertilized mice and may play a role in both male (Pintado et al., 2003) and female reproductive systems (Schmidt et al., 2003). There is a significant upregulation of NK-1R protein at full-term fetus and newborn infant with a peak at day 1, and it downregulates at day 8, which indicates that NK-1R may be involved in the mechanisms modulating the processes during labor and after birth. SP-IR has an opposite correlation with NK-1R protein expression in pregnancy and uterus after birth (Lavezzi et al., 2015). It correlates with our own previous study (Mehboob, 2017), in which it was proposed, based on our experimental work on sudden infant death syndrome, sudden fetal death victims, and controls (Mehboob et al., 2017), that SP expression is normally low in healthy fetuses but higher in neonates as compared to controls, and if *vice versa*, it may lead to sudden death. Furthermore, it was proposed that the expression of SP is variable, depending on the developmental stage. It is lower in adults and, if increased, it may lead to death (Mehboob, 2017). This study provided further evidence to strengthen our previous hypothesis. The possible reason is that SP is involved in cardiorespiratory control centers of the brain and has many important functions (Munoz et al., 2013; Mehboob et al., 2014). It regulates and controls the sleep–wake cycle, respiratory rhythm as well. If there is any disturbance in its regulation, it may cause fatal outcomes including death. NK-1R is a receptor of SP, and all

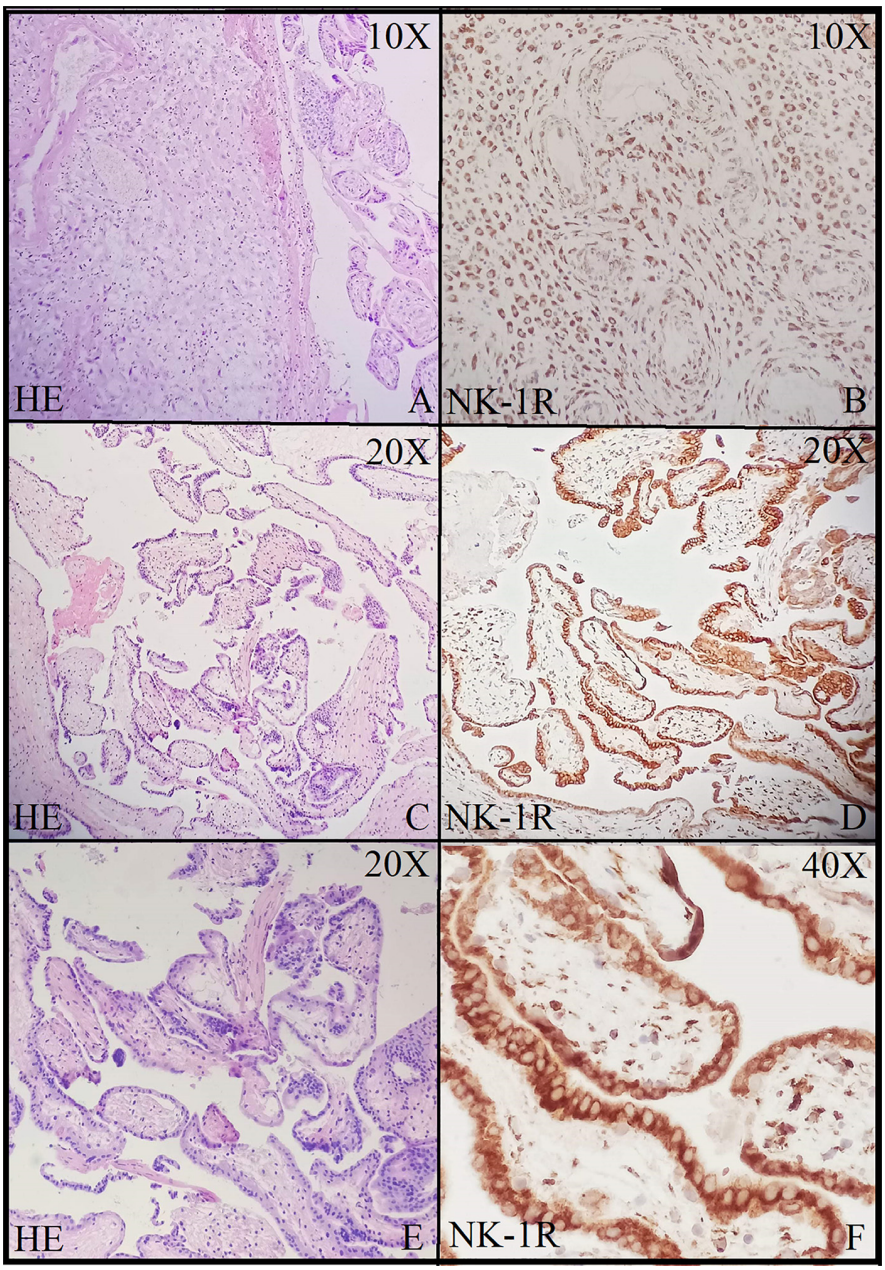


**FIGURE 3 |** Neurokinin-1 receptor (NK-1R) immunohistochemical staining in products of conception showing intense staining and positive expression in all the cells at 10× **(A,B)**, 20× **(C,D)**, and 40× **(E,F)**.

the functions of SP are only initiated, regulated, and modulated once SP binds with NK-1R. Here, in this study, we found an increased NK-1R expression, which shows that if SP is increased in the initial stage of pregnancy, it may cause spontaneous abortion or miscarriage; if at any fetal stage it may initiate the respiratory mechanisms, which is injurious, as the lungs have not yet started functioning and gaseous exchange is *via* feto–placental route. At full term, there is a need to expel the fetus, there may be a rise in SP expression, leading to birth of the baby, and the same SP is required for respiration through the lungs after birth. Our current result showing a strong NK-1R expression in placental tissue supports this point. Ebner and Singewald (2006) and Munoz et al. (2010) observed the expression of SP and NK-1R in all the cells of placenta, which is in line with our current and previous findings, e.g., SP levels are raised at term fetus, near birth, and soon after. We may speculate that SP is regulating the pregnancy and controlling the respiratory mechanisms, delivery, and cardiorespiratory controls.

The role of SP in stress and anxiety is already established (Zieglgansberger, 2019). It is released more in such conditions as

well as other nociceptive stimuli including pain (Fest et al., 2006). Stress-induced abortions may induce a rise in decidual tumor necrosis factor (TNF)-alpha and cause neurogenic inflammation. TNF-alpha is a possible stimulator for miscarriage (Joachim et al., 2001; Mowa et al., 2003). We already know that stress, pain, anxiety may lead to spontaneous miscarriage or preterm delivery as well. It shows that the possible mechanism may be *via* SP/NK-1R system. A study reported similar findings—that SP expression was elevated in full-term fetus and in newborn and may have played a role in cervical ripening and labor (Qian et al., 2018).

Pregnancy is a unique physiological process. During the early developmental stages, an immune rejection caused by fetal antigens is inhibited by the mother (Patak et al., 2000). The immune system has a very important role in pregnancy. During implantation, the endometrium makes the maternal–fetal connection and recruits T regulatory cells to the site, which is the local immune response (Wang et al., 2019). If there is any insufficiency in this process, it may cause spontaneous abortion (Mehboob et al., 2020). It may be speculated that increased immune reactions or hypersensitivity may cause immune rejection, leading to abortion. Immune dysfunction may have a significant role too.

I would like to mention one of our most recent clinical trials in which we have found that NK-1R antagonist aprepitant in combination with dexamethasone may improve the recovery of coronavirus disease 2019 (COVID-19) patients by improving respiratory recovery (Sha et al., 2017). Dexamethasone is already in medical use for strengthening lung functionality in premature deliveries. This is a very strong evidence in support of this current study because it shows that NK-1R is involved in respiratory physiology. It was observed that more adults were infected with COVID-19 as compared to infants and children. It is also in line with our hypothesis and findings. As we discussed earlier that SP/NK-1R may be less expressed in the adult neuromodulatory system, but if it is enhanced in case of nociception, e.g., COVID-19 or another infection, it may lead to a decrease in respiratory function. But in infants, the SP/NK-1R expression is already enhanced due to increased respiratory needs, hence, an activation of the NK-1R system due to stress or nociception may not have an adverse affect.

Human immunoglobulin (IG) has been used widely for the treatment of abortion (Muyayalo et al., 2018). It may modulate the immune mechanisms (Guo et al., 2020) in such a way that it may tolerate the embryo. The immune dysfunction may be enhanced by IG + hCG drug therapy and, hence, improve pregnancy outcomes. It needs to be further explored [45]. In a study, the outcomes were improved by 60% after the treatment. IG + hCG may be suggested to increase the rate of successful pregnancy by modulation of the immune function [45]. The limitations of the study include its small sample size and absence of a control group, which is impossible and immoral due to ethical reasons. As it was not possible to have a control tissue, we used placental tissue and have compared the results with our previous studies. Here, we suggest NK-1R antagonist in addition to the IG + hCG to diagnose and treat spontaneous abortion.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Lahore Ethics review board. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

All authors have contributed to the study in writing, planning, immunohistochemistry, microscopy and final approval.

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REVIEW
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# Research Advances on Therapeutic Approaches to Congenital Central Hypoventilation Syndrome (CCHS)

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Congenital central hypoventilation syndrome (CCHS) is a genetic disorder of neurodevelopment, with an autosomal dominant transmission, caused by heterozygous mutations in the *PHOX2B* gene. CCHS is a rare disorder characterized by hypoventilation due to the failure of autonomic control of breathing. Until now no curative treatment has been found. PHOX2B is a transcription factor that plays a crucial role in the development (and maintenance) of the autonomic nervous system, and in particular the neuronal structures involved in respiratory reflexes. The underlying pathogenetic mechanism is still unclear, although studies *in vivo* and in CCHS patients indicate that some neuronal structures may be damaged. Moreover, *in vitro* experimental data suggest that transcriptional dysregulation and protein misfolding may be key pathogenic mechanisms. This review summarizes latest researches that improved the comprehension of the molecular pathogenetic mechanisms responsible for CCHS and discusses the search for therapeutic intervention in light of the current knowledge about *PHOX2B* function.

**Keywords: autonomic dysregulation, breathing disorder, congenital central hypoventilation syndrome, CCHS, PHOX2B, polyalanine expansions, desogestrel**

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## INTRODUCTION

Sudden infant death syndrome (SIDS), Rett syndrome (RTT), and congenital central hypoventilation syndrome (CCHS) are human diseases that share developmental defects in the neural circuits that control breathing (**Box 1**) (Ramirez et al., 2018, 2020; Trang et al., 2020). Breathing is under the control of three linked networks of brainstem neurons responsible for respiratory rhythm generation (Benarroch, 2018, and references therein): the pre-Bötzinger complex (pre-BötC), that drives inspiration, the retrotrapezoid/parafacial respiratory group (RTN/pFRG), that controls active expiration and plays an important role in central chemosensitivity (Guyenet et al., 2019; Pisanski and Pagliardini, 2019), and the post-inspiratory complex (PiCo) controlling post-inspiration (Anderson et al., 2016). The RTN contains glutamatergic neurons, expressing *VGlut2, NK1R, Neuromedin B,* and *PACAP* (Guyenet et al., 2019), whose development depends on the transcription factor paired-like homeobox 2B (PHOX2B), a master gene of the development of the autonomic nervous system (ANS) (Pattyn et al., 1999). Other neural structures are under the developmental control of PHOX2B, such as the locus coerules (LC), the catecholaminergic neurons of the nucleus of solitary tract (NTS) and of the

C1 groups, and all these neurons are also activated by hypercapnia, and participate in respiratory reflex (Brunet and Pattyn, 2002; Stornetta et al., 2006; Gargaglioni et al., 2010).

Congenital central hypoventilation syndrome (CCHS or Ondine's curse; MIM# 209880) is a rare neurological disorder characterized by the deficient autonomic control of breathing (Weese-Mayer et al., 2010; Trang et al., 2020). So far, although some drugs have shown some effects in improving ventilation (Straus et al., 2010; Schirwani et al., 2017), CCHS remains a severe breathing disorder without any effective pharmacological treatment.

*In vitro* and *in vivo* studies have contributed to the elucidation of the physiological role of the *PHOX2B* gene and the molecular consequences caused by its mutations (Di Lascio et al., 2018a). Despite an incomplete picture of the CCHS pathogenetic mechanisms, recent studies have suggested that the development of new therapeutic strategies may target the primary pathogenetic defect of CCHS, or by-pass it by pharmacological compensation. Moreover, the report of ventilation improvement in two CCHS patients using the progestin drug desogestrel as a contraceptive (Straus et al., 2010) has opened up new perspectives for therapeutic research.

In this review we will summarize the current knowledge about the molecular pathogenesis of CCHS and will discuss the recent progress and perspectives in the advancement of therapeutic research in the light of the new insights into the role of PHOX2B.

## CONGENITAL CENTRAL HYPOVENTILATION SYNDROME (CCHS): CLINICAL PRESENTATION, DIAGNOSIS, AND MANAGEMENT

Alveolar hypoventilation is the hallmark of the disease, caused by an abnormally reduced or absent ventilatory response to hypoxia and hypercapnia caused by the malfunctioning of PHOX2B-mediated regulation of autonomic respiratory control and chemosensitivity. Hypoventilation is generally more severe during sleep [especially during non-rapid eye movement (NREM) sleep] than during wakefulness (Weese-Mayer et al., 2010; Trang et al., 2020). Drugs that stimulate ventilation that have been tested so far are not effective (Weese-Mayer et al., 2010), and management is supported by lifetime assisted ventilation.

The CCHS-defining gene *PHOX2B* (Amiel et al., 2003; Weese-Mayer et al., 2003) encodes a transcription factor whose role as master gene in the development of ANS and of the neural structures involved in breathing control has been clearly defined (Pattyn et al., 1999, 2000). Mutations in the *PHOX2B* gene interfere with the development of the neuronal network that regulates $CO_2$ chemosensitivity and breathing (Dubreuil et al., 2008).

The age of presentation of CCHS is typically in newborns, but it may also be diagnosed later in childhood or adulthood [later-onset (LO) CCHS] (Weese-Mayer et al., 2005; Antic et al., 2006; Trochet et al., 2008b; Basu et al., 2017). The disease can be isolated (Di Lascio et al., 2018b; Bachetti and Ceccherini, 2020), or associated with a spectrum of non-respiratory symptoms, among which seizures (Binmanee et al., 2020) and other conditions that reflect a more global ANS dysfuntion, including cardiac arrhythmias and congenital heart disease (Lombardo et al., 2018; Laifman et al., 2020), ocular disorders, the aganglionic megacolon Hirschsprung's disease and neural crest tumors (reviewed in Di Lascio et al., 2018a,b; Broch et al., 2019; Bachetti and Ceccherini, 2020; Trang et al., 2020).

CCHS is rare: its estimated incidence is 1/148,000–1/200,000 live births (Trang et al., 2005; Shimokaze et al., 2015), with a total of about 1,300 genetically confirmed cases worldwide (Di Lascio et al., 2018a).

Trasmission of CCHS is autosomal dominant with variable expressivity and incomplete penetrance, and without any gender preference (Weese-Mayer et al., 2003; Bachetti and Ceccherini, 2020). Variable CCHS-like phenotypes have been reported associated with whole and partial gene deletions of *PHOX2B* as extensively reported (Jennings et al., 2012; Bachetti and Ceccherini, 2020).

The primary aim of CCHS management is to provide adequate ventilation and oxygenation, and three types of long-life ventilatory support [pressure-controlled ventilation via tracheostomy, non-invasive positive pressure ventilation (mask ventilation), or diaphragm pacing] are commonly used by CCHS patients, according to a number of factors, including the patient's age, the age of disease onset and ventilatory parameters. The pros and cons of the use of different ventilatory supports are well summarized in the recent published guidelines for diagnosis and management of CCHS (Trang et al., 2020). It is worth noting that the use of some of these supports always requires the presence of carers that monitor the correctness of ventilation and can change the settings, if required, with a great effort of the patient's families. Moreover, the management of patients with CCHS and the associated conditions of autonomic dysregulation can be more complex, and should include a global assessment of the digestive, cardiovascular, and ocular systems, and the investigation for tumors of neural crest origin (Weese-Mayer et al., 2017; Bishara et al., 2018; Di Lascio et al., 2018a; Maloney et al., 2018; Zaidi et al., 2018; Trang et al., 2020).

Neurocognitive deficits have been reported in CCHS children (Charnay et al., 2016; Zelko et al., 2018), and probably both the primary developmental damage and the chronic episodes of hypoxia–reoxygenation, potentially occurring during nocturnal assisted ventilation but also during wakefulness, contribute to neurological outcome. It has recently been reported that these conditions may cause the overproduction of reactive oxygen species (ROS) (Degl'Innocenti et al., 2018), inducing oxidative stress, cellular damage and eventually activating apoptotic pathway (Rahal et al., 2014). This effect has been already demonstrated in other breathing disorders characterized by intermittent hypoxia such as sudden infant death syndrome (SIDS), obstructive sleep apnea syndrome (OSAS), and Rett syndrome (Prandota, 2004; Lavie, 2015; Bebensee et al., 2017). According to the American Thoracic Society (ATS) recommendations, neuropsychological status should be assessed in all CCHS patients in order to early

identify and treat those individuals that would benefit from early interventions to ameliorate their cognitive abilities (Macdonald et al., 2020).

A *PHOX2B* mutation would be always required for a diagnosis of CCHS; however, in individuals with CCHS or phenotypes similar to CCHS and negative for *PHOX2B* mutations, homozygous mutations in the *MYO1H* gene (Spielmann et al., 2017), and in *LBX1* gene (Hernandez-Miranda et al., 2018) have recently been found. In particular, LBX1 cooperates with PHOX2B in the development of the retrotrapezoid nucleus (RTN) (see below), and its frameshift mutation interferes with this cooperativity, probably by blocking the recruitment of co-activator and/or a possible interaction with PHOX2B, thus changing the way their target genes are regulated (see below).

The variable phenotypes reported in CCHS patients carrying the same mutation also suggest the involvement of modifier genes of expressivity (Di Lascio et al., 2018a; Bachetti and Ceccherini, 2020). Mutations in genes other than *PHOX2B*, involved in the differentiation of neural crest cells (*RET*, *GDNF*, *BDNF*, *GFRA1*, *PHOX2A*, *HASH-1*, *EDN1*, *EDN3*, *BMP2*) or in oncogenes (*BRAF*) (Bolk et al., 1996; Amiel et al., 1998; Weese-Mayer et al., 2002, 2003; Sasaki et al., 2003; Fernández et al., 2013; Al Dakhoul, 2017) have been found in some CCHS individuals. Remarkably, some of them are PHOX2B target genes (Flora et al., 2001; Bachetti et al., 2005), but the pathogenic role of these genetic variants is unclear (de Pontual et al., 2007).

### *PHOX2B* Mutations in CCHS

The *PHOX2B* gene is located at chromosome 4 (4p13) and is composed of three exons. PHOX2B protein is a transcription factor of 314 aminoacids, with a conserved 60-residues DNA binding domain (homeodomain) and two short and stable stretches of 9 and 20 alanine residues in the C-terminus (**Figure 1**).

The most frequent mutations found in CCHS patients are in-frame triplet duplications of different lengths within the sequence stretch coding for the 20 alanine tract. The polyalanine (polyAla) repeat expansion mutations (PARMs) lead to the addition of 4–13 alanine residues (normal genotype 20/20, CCHS genotypes 20/24–20/33), and short expansions of five, six, and seven alanines (genotypes 20/25, 20/26, 20/27) are the most frequent. Previous studies have tentatively described a genotype-phenotype correlation between the length of the expansion and the severity of the respiratory phenotype (Matera et al., 2004; Weese-Mayer et al., 2017; Di Lascio et al., 2018a,b; Bachetti and Ceccherini, 2020), but this relationship is variable (Trang et al., 2020). Homozygous individuals for the shortest polyalanine expansion (+ 4 alanines) have been described, whose phenotypes ranged from asymptomatic to severely affected (Trochet et al., 2008a; Repetto et al., 2009; Kwon et al., 2011; Chuen-im et al., 2014).

The minority of CCHS patients have heterozygous missense (MS), nonsense (NS) and frameshift (FS) mutations in exon 1, 2, or 3 [defined non-PARM mutations (NPARMs)]. The most frequent NPARM mutations are FS mutations that lead to a truncated or an elongated C-terminal region (reviewed in Weese-Mayer et al., 2017; Di Lascio et al., 2018b; Bachetti and Ceccherini, 2020), and can be associated with more severe syndromic forms, in which CCHS occurs together with HSCR and/or neuroblastoma (Matera et al., 2004; Berry-Kravis et al., 2006; Di Lascio et al., 2018b). Recently, it has been described a case of CCHS due to a combination of a PARM (+ 4 alanine) and a NPARM mutation, inherited from asymptomatic parents, each heterozygous for these *PHOX2B* variants (Sivan et al., 2019).

Polyalanine contractions have been reported in few CCHS or HSCR patients (Weese-Mayer et al., 2010; Di Zanni et al., 2017) and have also been found in a small percentage (1–1.5%) of the general population. Therefore, these variants are not considered diagnostic of CCHS, although they may have predisposing effects in HSCR and SIDS (Bachetti and Ceccherini, 2020, and references therein). A similar role has been hypothesized for the higher frequency of synonymous SNPs in *PHOX2B*, associated with OSAS (Lavezzi et al., 2013) and SIDS (Weese-Mayer et al., 2004), the reduced *PHOX2B* expression with protein dislocated in the cytoplasm in neurons from SIDS specimens (Lavezzi et al., 2012), and a complete *PHOX2B* gene deletion in an apparent life threatening event (ALTE) patient (Jennings et al., 2012).

## MODELING CCHS: *IN VIVO* AND *IN VITRO* APPROACHES

Most of our knowledge about CCHS pathogenesis derives from the use of (i) neuroblastoma cell lines, (ii) animal models, with the possibility to derive tissue explants and organotypic cultures, and (iii) postmortem tissue analysis and biopsies. Although all of these approaches show some limitations, they have enabled significant strides toward understanding the pathological processes of the disease. Here we summarize the most important findings on PHOX2B physiological function and the molecular defects caused by its mutations, derived from *in vivo* and *in vitro* models of CCHS.

### *PHOX2B* in Neuronal Development and Cell Function

The *PHOX2B* gene is considered a "master selector gene" in the development of the ANS and the neural structures that are involved in breathing control and respiratory reflexes (Pattyn et al., 1999, 2000). In particular, central noradrenergic and hindbrain visceromotor neurons generation and maintenance, at least up to the late embryonic stages (Coppola et al., 2010; Fan et al., 2011), depends on PHOX2B, along with its paralog PHOX2A. In adult rats, *Phox2b* is expressed in several brainstem structures (Kang et al., 2007), although its exact role in adulthood is still to be defined. In particular, hypercapnia-sensitive neurons of the brainstem (Stornetta et al., 2006), including the neurons in the nucleus of the tractus solitarius (NTS) (Pattyn et al., 1997) and in the carotid bodies, express *Phox2b*. Moreover, $Phox2b^{+}/VGlut2^{+}/Nmb^{+}$ glutamatergic neurons located on the ventral surface of the medulla oblongata (Stornetta et al., 2006; Shi et al., 2017), below the facial motor nucleus, form the retrotrapezoid nucleus (RTN). This structure is involved in integrating peripheral and central chemoception (Dauger et al., 2003; Mulkey et al., 2004; Takakura et al., 2006; Guyenet and Bayliss, 2015), and mediating most of the ventilatory

**BOX 1 |** SIDS, RETT, and CCHS main features.

*Sudden Infant Death Syndrome (SIDS)*

- SIDS is a complex heterogeneous disorder referred to death during sleep in seemingly healthy infants < 1 year old occurring suddenly and unexplained.
- It is mainly due to respiratory failure during sleep (arouse in response to altered oxygen or $CO_2$ levels), and abnormalities in a number of physiological functions and systems (neurological, cardiovascular, respiratory, gastrointestinal, endocrine, metabolic, immune and genetic) have also been reported.
- Its occurrence is estimated in 0.40 SIDS deaths per 1,000 live births.
- No diagnostic features are available up today. SIDS is due to a variety of factors conceptualized in the Triple Risk Model: 1. A critical developmental period in homeostatic control (Central nervous system and Immune system); 2. Vulnerable infant (race or exposure to alcohol or tobacco during pregnancy, genetic polymorphisms, low birth weight, prematurity). 3. Exogenous stressor(s) (overheating, tobacco smoke, upper respiratory tract infection, bed sharing, and prone sleeping position). Death occurs when they are expressed simultaneously.
- One of the main hypothesis is that SIDS is associated with defects in medullary homeostatic control ("brainstem hypothesis"), characterized by malfunctioning neurotransmitter networks, including catecholamines, neuropeptides, serotonin and its receptors, glutamate, brain-derived neurotropic growth factor (BDNF), and some cytokine systems. In particular, abnormalities in the main serotonergic centers [raphe, extra raphe, and ventral (arcuate) populations of 5-HT neurons] and their projection sites (dorsal motor nucleus of the vagus and the nucleus of the solitary tract) and lower expression of brainstem 5-HT, tryptophan hydroxylase-2 (TPH-2), and 5-HT receptor have been reported in SIDS.

*RETT Syndrome*

- RETT syndrome is a X-linked neurological disorder due to mutation in the *MeCP2* gene, whose symptoms appear 5-18 months after birth.
- Its incidence is 1/10,000 of female births.
- RETT girls show developmental delay, motor impairment, sleep problems, seizures, breathing and feeding dysfunction.
- Breathing dysfunction during wakefulness includes episodes of hyperventilation and irregular breathing and episodes of breath-holding. Respiratory disturbance during sleep includes episodes of obstructive sleep apneas (OSAs).
- Mouse model revealed defects in generation of central respiratory rhythm, including reduced ventilatory response to $CO_2$, and in the development of central noradrenergic (locus coeruleus) and serotonergic neurons.
- Respiratory instability (breathhold events) is also associated with deficient GABA-ergic transmission in the pontine Kolliker Fuse region (KF), the nucleus tractus solitarius (NTS), LC and ventrolateral medulla.
- Oxidative stress and lung inflammation are also hallmarks of the syndrome.

*Congenital Central Hypoventilation Syndrome (CCHS)*

- CCHS is a neurodevelopmental disorders due to heterozygous polyalanine expansion mutation (PARM) in the *PHOX2B* gene, a transcription factor involved in the development of the autonomic nervous system (ANS), including the neuronal structures that control breathing and integrate respiratory reflexes.
- Its estimated incidence is 1/148,000-1/200,000 live births (about 1,300 genetically confirmed cases worldwide).
- It is usually present at birth but late-onset (childhood and adulthood) cases have been diagnosed.
- Alveolar hypoventilation, due to reduced or absent ventilatory response to hypoxia and hypercapnia, is more severe during NREM sleep than during wakefulness.
- Respiratory phenotype in CCHS is mainly due to developmental defects in retrotrapezoid nucleus (RTN), a structure that integrates peripheral and central chemoreception, and that is missing in Phox2b/+7 alanine knock-in mouse model of CCHS. Defects in other areas, such as locus coeruleus (LC) have been reported in post-mortem brains of CCHS patients.
- Associated to CCHS, a spectrum of non-respiratory symptoms, including seizures, cardiac arrhythmias, congenital heart disease, ocular disorders, Hirschprung's disease and neural crest tumors (neuroblastoma) reflects a more global ANS dysfunction.

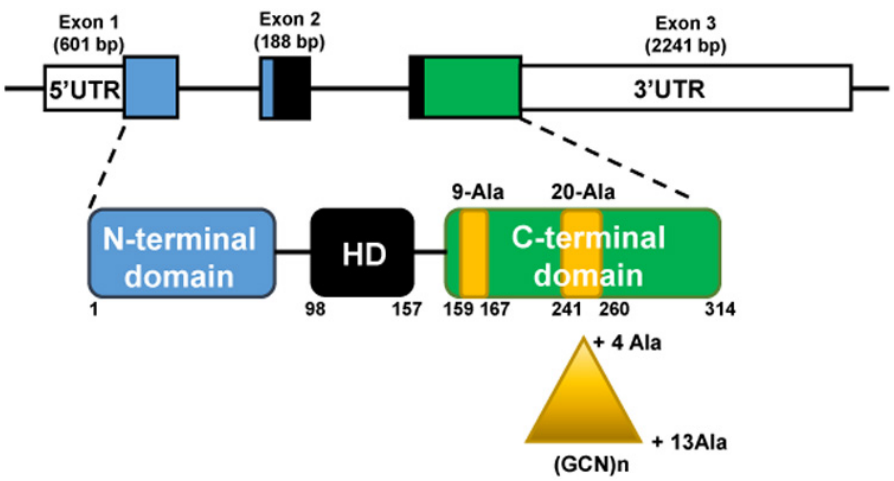


**FIGURE 1 |** The *PHOX2B* gene **(top)** and protein **(bottom)**. The gene is composed of three exons; the protein presents an homeodomain (HD, black box) and 9 and 20 residues poly-alanine tracts (yellow boxes) in the C-terminus part. Triangle indicates triplet expansion of the 20 poly-alanine coding sequence, giving rise to the 4–13 polyalanine (polyAla) repeat expansion mutations (PARMs). The numbers indicate PHOX2B amino acid residues.

reflex to hypercapnia, particularly during NREM sleep (Guyenet et al., 2016, 2019). The dependence on *Phox2b* expression of all these structures comes from the observation that they are missing in *Phox2b* knock-out mice (Pattyn et al., 1999; Dauger et al., 2003).

PHOX2B, by means of its homeodomain region and the C-terminal domain, forms homo- and heterodimers with other homeoproteins, including its paralog PHOX2A (Adachi et al., 2000; Di Lascio et al., 2016a). The C-terminal domain containing the two polyalanine stretches and in which the PARMs and the majority of NPARMs mutations are located is also involved in DNA binding affinity and solubility of the protein (Di Lascio et al., 2016a).

So far, several hypotheses have been proposed to find a role for polyalanine tracts (single amino acid repeats), among which flexible spacer linking functional protein domains, drivers of protein conformation, protein-protein interactions, and DNA-binding, thus modulating transcription factor activity (Di Lascio et al., 2018a). Indeed, it has been demonstrated that the presence of the 20-alanine stretch is important for PHOX2B transcriptional activity (Radó-Trilla et al., 2015), but it is not required for protein localization and its ability to form dimers (Di Lascio et al., 2013, 2016a).

## Developmental Defects in Mouse Genetic Models and CCHS Patients

To investigate the developmental defects occurring in CCHS patients, several mouse models have been generated (reviewed in Amiel et al., 2009; Moreira et al., 2016; Di Lascio et al., 2018a). Constitutive and conditional knock-in (KI) models, carrying both PARM and NPARM *PHOX2B* mutations, show impaired $CO_2$ chemosensitivity and the selective deletion of the RTN as a common trait, thus indicating it as the main cause of the respiratory phenotype. However, it is unlikely that the respiratory deficits observed in some patients during wakefulness could be explained by RTN absence or defects. Indeed, it has been hypothesized (Tremoureux et al., 2014) that, in wakefulness, activation of motor areas (i.e., cortex) may compensate for the defect in brainstem centers controlling autonomic breathing. In CCHS patients it has been reported the existence of a "resource competition" (Sharman et al., 2014) between control of breathing by cortical areas and other cortical functions, that require mental concentration (such as watching television, video gaming, and studying) resulting in increased hypoventilation in CCHS patients, and indeed mechanical ventilation during wakefulness ameliorates cognitive performances. Until now, the existence and location of RTN have been only tentatively defined in humans (Rudzinski and Kapur, 2010; Lavezzi et al., 2012) and the recent analyses on post-mortem brains of CCHS patients did not confirm defects in any RTN-like structure (Nobuta et al., 2015). Conditional KI mice, carrying a NPARM *PHOX2B* mutation, shed light in filling the gap between mice and human manifestation of the disease, indicating that the chemosensory control of breathing not only depends on RTN function, but also on noradrenergic neurons of *locus coeruleus* (LC), a neuronal structure that in +7 Alanine KI animals apparently developed normally (Dubreuil et al., 2008; Nobuta et al., 2015; Moreira et al., 2016). In NPARM KI mice, the mutation inhibits LC precursors differentiation, in addition to the loss of the RTN, facial nerve nucleus and intestinal aganglionosis. *PHOX2B* mutations therefore prevent neuronal development at different stages with variable outcomes (from complete loss to incomplete differentiation), and it is likely that both RTN loss and LC impairment contribute to the respiratory deficits of CCHS patients. Consistently, LC defects have recently been confirmed in two CCHS cases with different *PHOX2B* mutations (one PARM and the same NPARM of conditional KI animals) (Nobuta et al., 2015).

The existence of a LC-preBötzinger complex circuit controlling breathing has recently been demonstrated by Liu et al. (2020) by showing that selective stimulation of $Phox2b^+$ LC neurons enhanced basal ventilation in conscious mice, that $Phox2b^+$ LC neurons lesions by genetic manipulation reduced hypercapnic ventilatory responses, and that LC neurons are physically connected, by axons projection, to the complex. LC defects are also an hallmark in models of Rett syndrome (Roux et al., 2010), that share with CCHS respiratory defects, although more severe during wakefulness than during sleep (Weese-Mayer et al., 2008; Ramirez et al., 2020).

Recently, it has been shown that mutant Phox2b may induce secondary/non-cell autonomous defects of key brainstem respiratory neurons (Alzate-Correa et al., 2020). Indeed, the expression of NPARM *PHOX2B* mutation in $Phox2b^+$ non-respiratory progenitor cells such as visceral motor neuron progenitors, induced a severe neonatal apnea along with a significant loss of neurons directly deriving from that specific progenitor domain but also from respiratory neural structures, such as RTN and preBötC, embriologically unrelated to that progenitor domain.

CCHS patients also have cardiovascular, gastrointestinal and ocular deficits that indicate additional brainstem defects, consistent with the recent brain imaging studies (Harper et al., 2015).

Recently, the role of astrocytes in the chemosensitive response (paracrine hypothesis) has been postulated (Guyenet et al., 2019, and references therein). In particular, it has been reported that PHOX2B-derived astrocytes play a role in chemosensory control of breathing (Czeisler et al., 2019), especially in the adult, by maintaining a functional $O_2$ chemosensitive response, adequate sleep homeostasis and by ensuring synaptic integrity of neurons in RTN. It is then plausible to hypothesize that *PHOX2B* mutations may have consequences also in the development of this group of astrocytes. This finding adds a level of complexity in the understanding of neuronal control of breathing, but unraveling the molecular mechanism of such a control may represent a new challenge into the comprehension of CCHS pathogenesis.

## Mutant PHOX2B Proteins Show Altered Properties and Functional Activities

Data from experimental models of CCHS suggest that different pathogenetic mechanisms (loss-of-function, dominant-negative,

and toxic effects of the mutant proteins) may contribute to explain the entire spectrum of CCHS. These mechanisms are recapitulated in **Figure 2**, and extensively reviewed (Di Lascio et al., 2018a, and references therein). Protein misfolding and transcriptional dysregulation are among the most studied.

Protein misfolding has been extensively studied *in vitro:* both PARM and NPARM mutations form oligomers (Di Lascio et al., 2018a) and recently, it has also been reported a tendency of the + 7 alanine variant to form fibrils (Pirone et al., 2019). In cell models, only longer polyalanine expansions caused cytoplasmic aggregation (Di Lascio et al., 2018a, and references therein) and it has been proposed that the polyalanine tract may play a role in the cytoplasmic mislocalization of transcription factors containing poly(A)-tracts, acting as a nuclear export signal (NES) (Li et al., 2017), by means of interaction with the elongation factor 1A1 (eEF1A1). The reduction of *eEF1A1* restores the nuclear localization of poly(A)-containing transcription factors and their transcriptional activities. Moreover, a fraction of PHOX2B wild-type protein is sequestered by the mutant protein in the cytosol, thus having a dominant negative effect on the localization and activity of the normal protein (Parodi et al., 2012; Di Lascio et al., 2013).

Conversely, frameshift mutations retain nuclear localization, but formed inclusions and accumulate in the nucleoli (Di Lascio et al., 2018b; Ye et al., 2019). It is worth noting that the *in vivo* aggregation of PHOX2B protein has not been reported so far, and so the role of aggregates and their toxic effects in CCHS is unclear. In favor of the aggregation theory, there is the finding that anesthetic agents can promote LO-CCHS, by inducing aggregation and mislocalization of PHOX2B variants via the endoplasmic reticulum (ER) unfolded protein response (UPR) (Basu et al., 2017; Coghlan et al., 2018).

Studies of cell models of CCHS indicate that, along with protein misfolding, transcriptional dysregulation may be another important pathogenetic mechanism (**Figure 2**). PARMs and NPARMs both show decreased DNA binding capability and transcriptional activity (**Figure 2A**), and mutant proteins reduce the activation of some PHOX2B target genes (Nagashimada et al., 2012; Di Lascio et al., 2013, 2018b), as reviewed in Di Lascio et al. (2018a). Moreover, PHOX2B also transactivates its own promoter (Cargnin et al., 2005), and the mutations may also interfere with this auto-regulation mechanism thus contributing to a reduction of the amount of normal PHOX2B protein.

It has also been reported that frameshift mutations aberrantly regulate some target genes (**Figure 2B**), such as *SOX10* and the glial acidic fibrillary protein (*GFAP*) (Nagashimada et al., 2012; Di Lascio et al., 2018b).

PHOX2B forms homodimers and heterodimers with PHOX2A (Adachi et al., 2000; Di Lascio et al., 2016a; Moreira et al., 2016) and dimerization is crucial for DNA-binding and transcriptional activity (**Figure 2C**). Alanine expansions progressively reduce the formation of dimers and PARM mutants interact weakly with normal protein (Di Lascio et al., 2016a); some of the observed dominant-negative effects are likely to be caused by the aberrant interactions with transcriptional co-activators or co-repressors (**Figure 2D**; Wu et al., 2009; Reiff et al., 2010).

It is interesting to note that PHOX2B mutants partially form heterodimers with PHOX2A retaining partial transcriptional activity, and they do not interfere with the localization and transcriptional activity of PHOX2A (Di Lascio et al., 2016a). This mechanism can also explain the CCHS cases in which a mutated partner of PHOX2B (namely LBX1) can interfere with the correct recruitment of co-activator necessary for the correct expression of PHOX2B target genes (Hernandez-Miranda et al., 2018). In brief, *PHOX2B* mutations cause a combination of loss-of-function, dominant-negative, and, gain-of-function effects and the entity of the transcriptional dysregulation is gene specific. Therefore, it is likely that the number and importance of the dysregulated target genes depends on the different types of mutations and this eventually determines the severity of the disease.

However, only a few PHOX2B regulated genes have been identified so far (**Figure 3**): tyrosine hydroxylase (*TH*) and dopamine-beta-hydroxylase (*DBH*), (Lo et al., 1999; Adachi et al., 2000); *PHOX2A* (Flora et al., 2001); *TLX2* (Borghini et al., 2006; Borghini et al., 2007); *RET* (Bachetti et al., 2005); *SOX10* (Nagashimada et al., 2012); *ALK* (Bachetti et al., 2010), and *PHOX2B* itself, because its expression depends on an auto-regulatory mechanism (Cargnin et al., 2005). To better understand the entire spectrum of CCHS, the discovery of all PHOX2B target genes is mandatory.

## THERAPEUTIC RESEARCH BASED ON ADVANCES IN OUR UNDERSTANDING OF CCHS

The diagnosis and treatment of CCHS patients have made great progress after the discovery of *PHOX2B* as the disease causing gene; on the other hand its role as a regulator of the development of ANS arised some concerns regarding the possibility to explore a pharmacological intervention in CCHS. The failure of the development of the neuronal structure driving the chemosensitive response to hypoxia and hypercapnia in CCHS transgenic mice models and possibly in CCHS patients (i.e., the RTN), has been considered an insurmountable obstacle toward a therapeutic intervention, supported by the findings that damages to these and other neuronal structures can progressively occur (Harper et al., 2015), probably due to postnatal hypoxic and hypercapnic episodes or to the persistence of the detrimental effects of *PHOX2B* mutations in vulnerable cell populations.

A residual cardiorespiratory response has been reported in some younger CCHS patients, an observation that can be explained by the fact that the loss of RTN described in a mouse models with a + 7 alanine mutation is not complete, but limited to 70% of the cells (Dubreuil et al., 2008). Moreover, it cannot be excluded that other chemosensitive areas are activated to compensate for the loss of RTN.

These findings pave the way for the development of therapeutic strategies even after birth, as supported by studies of other neurodevelopmental disorders (Castrén et al., 2012; Matagne et al., 2017).

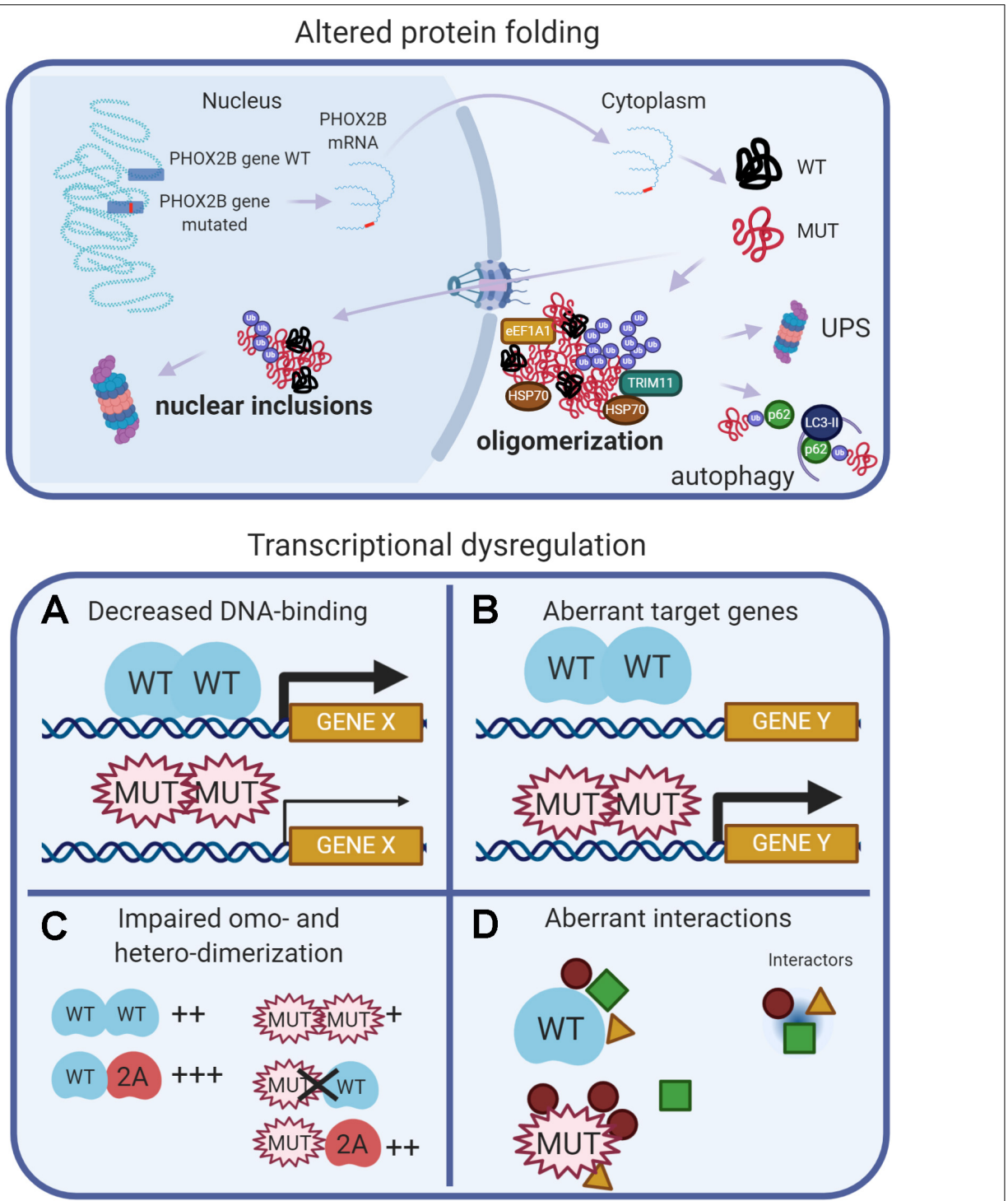


**FIGURE 2 |** (Created with BioRender.com). Molecular basis of CCHS. The upper panel shows that, in CCHS, mutations affect one copy of the *PHOX2B* gene. Transcription and translation give rise to two proteins, wild-type and mutated. Misfolded mutant proteins form aggregates in the cytoplasm, also sequestering wild-type proteins, and may be bound by molecular chaperones (heat shock proteins, HSPs) in an attempt to refold them. Alternatively, unsuccessful folding can facilitate their ubiquitination (Ub), a process involving ubiquitin ligases such as TRIM11, and recognition by the proteolytic machinery (UPS, Ubiquitin Proteasome System). Autophagy can participate in the removal of PHOX2B protein aggregates. A fraction of the mutant proteins can enter the nucleus, aggregate and form nuclear inclusions or induce transcriptional dysregulation (lower panels) by: **(A)** decreasing DNA binding, limiting PHOX2B transcriptional activity; **(B)** inducing aberrant genes expression; **(C)** decreasing the formation of homodimers and the interaction with the paralog PHOX2A; **(D)** aberrant interactions with transcriptional cofactors; some of these might be inappropriately enhanced, whereas others might be lost or unchanged.

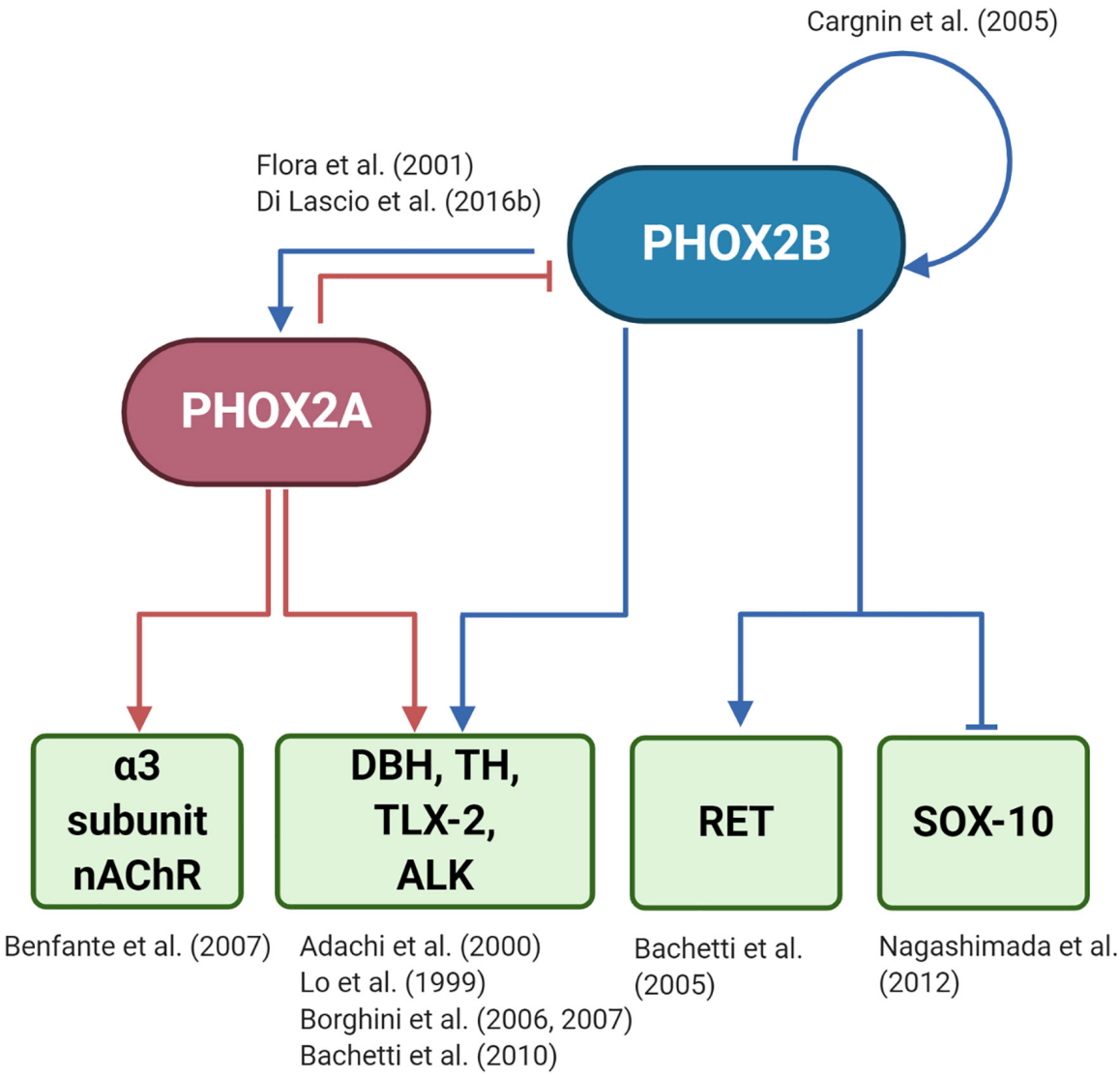


**FIGURE 3 |** (Created with BioRender.com) Target genes regulated by PHOX2B and PHOX2A. The diagram shows that PHOX2B regulates itself (Cargnin et al., 2005). Expression of the paralog gene *PHOX2A*, which is important for the expression of pan-autonomic genes as the α3 subunit of the ganglionic type nAChR (Benfante et al., 2007), depends on PHOX2B (Flora et al., 2001). PHOX2A, in turn, negatively regulates *PHOX2B* (Di Lascio et al., 2016b). Tyrosine hydroxylase (*TH*) and Dopamine-Beta-Hydroxylase (*DBH*), the rate-limiting enzymes of catecholamine biosynthesis (Lo et al., 1999; Adachi et al., 2000), TLX-2 (Borghini et al., 2006, 2007), a transcription factor involved in the development of enteric nervous sytem, and ALK (Bachetti et al., 2010), a tyrosine kinase whose mutations are associated with neuroblastoma, are regulated by both PHOX2 proteins. *RET* that is the most important gene driving the complex inheritance of Hirschsprung's disease (HSCR), is indirectly regulated by PHOX2B (Bachetti et al., 2005), as *RET* promoter does not contain a PHOX2B responsive element. The inhibition of *SOX10* by PHOX2B is critical for the differentiation of bipotential neural crest derived progenitors toward neuronal lineage (Nagashimada et al., 2012).

Another unexplored aspect of CCHS is how it can progress with age, in particular with respect to autonomic or cognitive functions; we can hypothesize that tuning the expression of *PHOX2B* and its target genes, as done by using retinoic acid (Di Lascio et al., 2016b), may divert the natural history of the disease. In this sense, a very recent study showed that forcing Phox2a/2b expression, in the LC of aged rats results in increased neurogenesis in the hippocampal dentate gyrus, increased norepinephrine levels in the striatum, and improved cognitive behavior, thus suggesting that Phox2a/2b play an important role in recovering noradrenergic and dopaminergic neurons function in aged animals (Fan et al., 2020).

In these perspectives, we report therapeutic approaches that have been exploited, *in vitro*, in order to reduce the damage caused by aberrant function of mutant PHOX2B. Furthermore, the serendipitous observation in two CCHS patients that chemosensitivity can be partially restored upon treatment with the progestin desogestrel has strong proof of concept value for a therapeutic approach in CCHS, at least in terms of relieving respiratory symptoms.

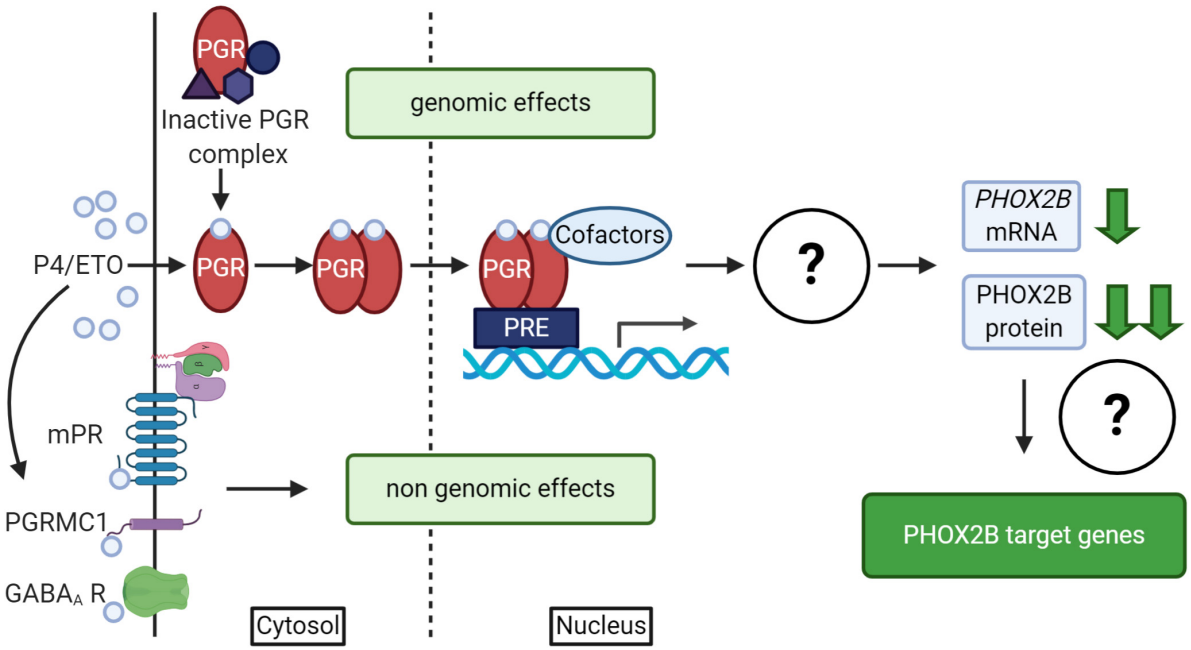


**FIGURE 4 |** (Created with BioRender.com). Schematic diagram of progesterone (P4)/progestins signaling pathways. Genomic effects of P4/ETO are mediated by nuclear progesterone receptor (PGR) which is inactive in the cytosol until progesterone binding. After P4 binding, it form dimers, translocates to the nucleus and dimers bind to Progesterone Responsive Element (PRE) within gene promoters and regulate the expression of target genes. The scheme also shows that the activation of PGR receptor by ETO is able to induce *PHOX2B* down-regulation by unknown mechanisms (Cardani et al., 2018), with possible effects on the expression of some PHOX2B target genes. Non-genomic effects of P4/ETO are mediated by the binding of P4 to cognate membrane receptors (mPR and PGRMC1) or to those belonging to other neurotransmitters (such as $GABA_A$ receptors).

## *In vitro* Studies

Based on *in vitro* studies on the mechanisms of CCHS pathogenesis summarized in **Figure 2**, the main goal of current therapeutic research, at the molecular level, is to hamper the toxic effects of mutant PHOX2B, focussing on molecules that can re-establish PARM-bearing mutant proteins correct localization and function, not only limited to CCHS (Di Zanni et al., 2011). As polyalanine expansions induce the formation of protein aggregates (Di Zanni et al., 2011; Pirone et al., 2019), possible target for pharmacological treatments are the pathways involved in the control of the protein quality (i.e., proteasome, autophagy and heat-shock pathways) with the aim to induce their activity to remove mutated proteins (Bachetti et al., 2007; Di Zanni et al., 2011, 2012; Parodi et al., 2012), as shown for geldanamycin and 17-AAG. These molecules, by recovering folding and correct localization to the nucleus, limited the dominant-negative effect of mutant PHOX2B protein on wild-type protein (Di Lascio et al., 2013). An alternative degradative pathway to reduce PHOX2B protein aggregates is mediated by autophagy (Bachetti et al., 2007; Di Zanni et al., 2012). Antioxidant therapies may also be beneficial in relation to the increase in ROS found in CCHS patients (Degl'Innocenti et al., 2018); curcumin, a major component of turmeric (*Curcuma longa*), promoted PHOX2B refolding (Di Zanni et al., 2012) without inducing the expression of molecular chaperones. It can be reasonable to hypothesize that different pathways (reviewed in Carvalho et al., 2016), based on its potent anti-inflammatory and anti-oxidant activities, may be involved.

The recent findings of the involvement of eEF1A1 in the mislocalization of polyalanine expanded protein into the cytoplasm, make this factor a new druggable target for poly(A) diseases (Li et al., 2017).

Another level of therapeutic intervention relies on the fact that many genes are regulated by PHOX2B, and those genes might be potential target for treating the disease. Eventually, the disease has to be considered the result of the aberrant expression (absent, down- or up-regulation expression) of PHOX2B target genes.

Unfortunately, very few genes are known to be PHOX2B targets (**Figure 3**), despite the fact that filling this gap is really urgent.

## *In vivo* Studies

### The Use of Progestins in CCHS: The Case of Desogestrel

In 2010, Straus et al. reported the fortuitous observation that two female patients (respectively, with 20/25 and 20/26 genotypes) underwent a partial recovery of chemosensitivity and increased ventilation when using the progestin drug desogestrel (13-ethyl gonanes family) for contraceptive purposes (Straus et al., 2010), although the magnitude of improvement was not sufficient to replace assisted ventilation (Straus and Similowski, 2011). This effect was not replicated on another CCHS patient (Li et al., 2013).

It is well known that progesterone, besides the reproductive and neuroprotective effect (Schumacher et al., 2014), powerfully stimulates respiration; indeed it has been used for the treatment of both adult apnea and of apneic pre-term neonates (Bairam et al., 2019, and references therein). Progesterone induces both genomic and non-genomic effects (Singh et al., 2013; Stanczyk et al., 2013; Schumacher et al., 2014) by the activation of nuclear (PGR) or membrane (mPR) receptors (Brinton et al., 2008; **Figure 4**); it has been shown that both receptors have a role in the modulation of chemoreflex response and respiratory control particularly during sleep (Bairam et al., 2019, and references therein). The exact molecular mechanism underlying this pharmacological effect is unknown; recently, *in vitro* studies showed that PHOX2B and desogestrel are molecularly linked demonstrating that the biologically active metabolite of desogestrel, 3-Ketodesogestrel (3-KDG; etonogestrel, ETO), modulates both wild type and mutant *PHOX2B* and the expression of its target genes via progesterone nuclear receptor PR-B (PGR) (Cardani et al., 2018). Remarkably, the expression of both wild-type and mutated PHOX2B is negatively regulated by 3-KDG (**Figure 4**). The exact mechanism and the molecular factor(s) involved are still unknown; however, it has been hypothesized that post-transcriptional mechanisms, including translation inhibition, could mediate 3-KDG effect on PHOX2B. As this process does not require the synthesis of new molecules, post-translational modifications (i.e., phosphorylation/de-phosphorylation), may be involved in mediating the effect of 3-KDG. In agreement with this hypothesis, it is known that monoubiquitylation of Phox2b/Phox2a by Rnf220/Zc4h2 complex is required for normal central noradrenergic neuron differentiation (Song et al., 2020).

PHOX2B downregulation by ETO does not apparently support the results reported by the clinical observation (Straus et al., 2010). In fact, 3-KDG decreases *PHOX2B* expression and haploinsufficiency is one of the mechanisms proposed in the insurgence of CCHS. However, as we described, respiratory defects may also depend on the gained toxic functions by mutant proteins (Di Lascio et al., 2018a), leading us to hypothesize that the positive clinical effect may be explained by the beneficial decreased level of the mutant PHOX2B, capable of counteracting the potential pathogenic effect of insufficient *PHOX2B* expression.

Data reported in Cardani et al. (2018) also showed that a slight PHOX2B decrease does not cause a down-regulation of all PHOX2B target genes, and in cell lines expressing *PHOX2A* and low levels of *PHOX2B*, progesterone may have the paradoxical effect of inducing tyrosine hydroxylase (Jensik and Arbogast, 2011), a well-known PHOX2B target gene. These findings suggest that *PHOX2B* expression level and the targeted cell-type may determine the positive or negative progesterone-mediated effects.

Among neuronal structures well known targets for progesterone are medulla oblongata, midbrain and diencephalon (**Figure 5**). In young mice, Pgr is expressed in different CNS structures (Quadros et al., 2007, 2008), including those areas that control breathing (Benarroch, 2018), but only in a few of those Pgr turned out to be co-expressed with PHOX2 proteins (**Table 1**; Kang et al., 2007; Quadros et al., 2008; Card et al., 2010).

Interestingly, the NTS, located in the dorsal medulla and known to be indispensable for the integration of sympathetic and respiratory responses to hypoxia and hypercapnia (Zoccal et al., 2014; Fu et al., 2019), is the only structure which co-expresses Phox2a, Phox2b, and Pgr (**Table 1**), and it has been reported that NTS neurons respond to progesterone and 3-KDG administration (Pascual et al., 2002; Loiseau et al., 2014; Joubert et al., 2016).

The reticular formation nuclei, such as the Periaqueductal Gray (Subramanian et al., 2008; Lopes et al., 2012), and the Parabrachial nucleus (Zuperku et al., 2019) are particularly interesting, because they co-express Pgr and Phox2a and it is known to play a role in breathing modulation (Card et al., 2010). Another structure that shows a Phox2a/Pgr restricted expression is hypothalamus, in particular the Dorsomedial hypothalamic nucleus (DMH), in caudal hypothalamus, and the Ventrolateral preoptic nucleus (VLPO), in rostral hypothalamus, both found to be important in the control of behavioral state (Saper et al., 2005). Moreover, VLPO controls the wake-sleep cycle regulation (Saper et al., 2005), whereas chemical stimulation of DMH has been associated to increased phrenic nerve stimulation (Tanaka and McAllen, 2008). The lateral area of hypothalamus contains $CO_2/H^+$ chemosensitive orexin neurons (Wang et al., 2018), and it has been reported that they contribute to the hypercapnic ventilatory response (Loiseau et al., 2019, and references therein), in a state-dependent manner (Rodrigues et al., 2019). These neurons have recently been involved in the desogestrel effect (Loiseau et al., 2014, 2018, 2019). In particular, data from Bodineau's group (Loiseau et al., 2014; Joubert et al., 2016) suggest that two distinct pathways, medullary and supra-medullary, are involved in the ETO ventilatory effects, excluding the involvement of RTN in the progestin effect.

By working on *ex vivo* mouse medullary-spinal cord preparations or *in vivo* newborn mice from both genders exposed to ETO, Loiseau et al. (2019) identified in orexin neurons the target of ETO-induced facilitation on respiratory frequency increase, following metabolic acidosis, and suggested that this was due to a non-genomic effect of desogestrel (Singh et al., 2013). The presence of PR receptors (nuclear and/or membrane) in orexin neurons is still unknown; however, the role of membrane progesterone receptor (mPR) could be excluded because progestins have little if any binding affinity for this membrane receptor (Giatti et al., 2016). Besides this, it has been hypothesized that ETO effect could be mediated by the modulation of other neurotransmitter systems. Indeed, progesterone and its metabolites act as allosteric modulator of $GABA_A$ receptor, potentiating GABA-induced chloride conductance (**Figure 4**), and of other Cys-loop family receptors (i.e., AChR, glycine, and 5-HT3), but also NMDA (NMDAR) and kainate receptors. Steroids, by binding NMDAR, potentiate the NMDA induced increase in respiratory frequency. However, whether synthetic progestin metabolites modulate $GABA_A$ receptor is still a matter of debate (Giatti et al., 2016; Joubert et al., 2016; Loiseau et al., 2018).

In conclusion, Loiseau et al. (2019) suggested that ETO induces the $CO_2/H^+$ chemosensitivity improvement in some CCHS patients by stimulating, or potentiating still functioning $CO_2/H^+$ chemosensitive central structures. However, potential

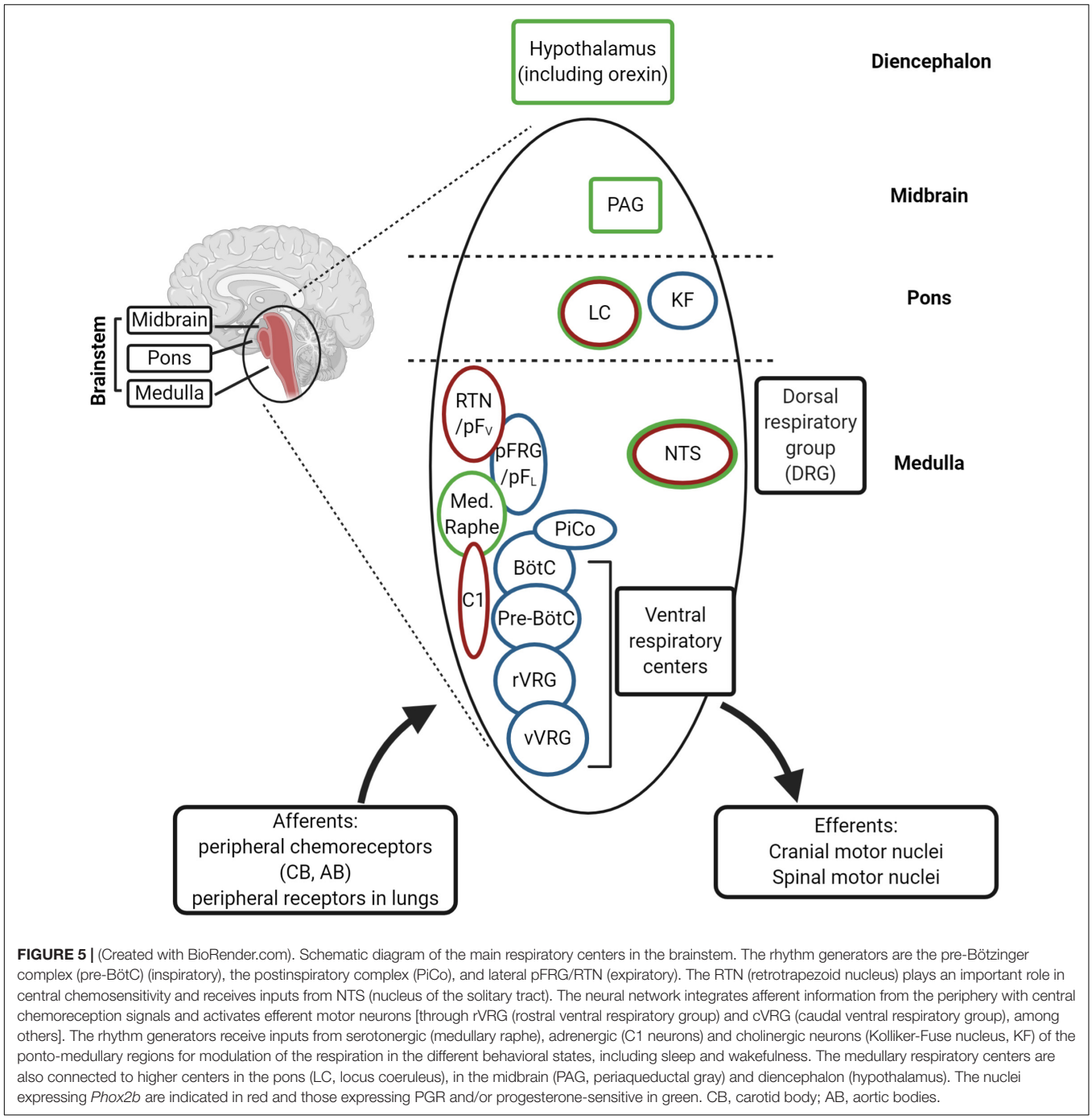


**FIGURE 5 |** (Created with BioRender.com). Schematic diagram of the main respiratory centers in the brainstem. The rhythm generators are the pre-Bötzinger complex (pre-BötC) (inspiratory), the postinspiratory complex (PiCo), and lateral pFRG/RTN (expiratory). The RTN (retrotrapezoid nucleus) plays an important role in central chemosensitivity and receives inputs from NTS (nucleus of the solitary tract). The neural network integrates afferent information from the periphery with central chemoreception signals and activates efferent motor neurons [through rVRG (rostral ventral respiratory group) and cVRG (caudal ventral respiratory group), among others]. The rhythm generators receive inputs from serotonergic (medullary raphe), adrenergic (C1 neurons) and cholinergic neurons (Kolliker-Fuse nucleus, KF) of the ponto-medullary regions for modulation of the respiration in the different behavioral states, including sleep and wakefulness. The medullary respiratory centers are also connected to higher centers in the pons (LC, locus coeruleus), in the midbrain (PAG, periaqueductal gray) and diencephalon (hypothalamus). The nuclei expressing *Phox2b* are indicated in red and those expressing PGR and/or progesterone-sensitive in green. CB, carotid body; AB, aortic bodies.

limitations of this work are that the study has been conducted in newborn wild type animals, whereas the two CCHS patients in Straus' study are adult, and the neuronal structures might be immature compared to adult humans. Indeed, in CCHS patients ETO potentiates also basal ventilation (Joubert et al., 2016; Loiseau et al., 2018) that may require the functioning of serotoninergic neurons within the medullary raphe nuclei, and modulation of $GABA_A$– and NMDA-mediated ventilatory regulations (Loiseau et al., 2018). Furthermore, the use of wild type animals has not allowed to explore whether orexin neurons stimulation still contributes to respiratory improvement in the presence of *PHOX2B* mutations.

The range of effective ETO concentration was very narrow and according to authors (Loiseau et al., 2019) the dose used in another unresponsive CCHS patient was inappropriate, thus explaining the observed unresponsiveness to desogestrel (Li et al., 2013). Altered ETO metabolism and/or impaired permeability to steroids of the blood-brain-barrier may be the cause (Stanczyk et al., 2013) and suggests that the effective desogestrel dose has to be personalized.

**TABLE 1 |** Brain distribution of Phox2a, Phox2b, and progesterone receptor (Pgr) immunoreactivity in adult rats.

| Adult rat (brain area) | Phox2a Card et al., 2010 | Phox2b Kang et al., 2007 | Pgr Quadros et al., 2007, 2008 |
|---|---|---|---|
| **Brainstem** | | | |
| **Medulla** | | | |
| Nucleus of the solitary tract (NTS) | + | + | + |
| Area postrema (AP) | + | + | – |
| Dorsal motor vagal nucleus (DMV) | ± | + | – |
| Inferior salivatory nucleus | + | – | – |
| Subjacent to the fourth ventricle | + | + | ± (vestibular nucleus) |
| **Ventrolateral brainstem (Medulla and pons)** | | | |
| Rostroventral medulla (C1 neurons) | + | + | – |
| Retrotrapezoid nucleus (RTN) | + | + | – |
| Superior salivatory nucleus | + | + | – |
| Nucleus ambiguus | – | + | – |
| **Pons** | | | |
| Locus coeruleus (LC) | + | – * | – |
| Parabrachial nucleus, lateral | – | –(rare) | + |
| Parabrachial nucleus, medial | + | –(rare) | + |
| Raphe pontis nucleus | – | – | + |
| Pedunculopontine tegmental nucleus | – | – | + |
| **Midbrain** | | | |
| Periaqueductal gray | + | – | + (not ventral) |
| A7 cell group | + | – | – |
| Substantia nigra, compact | – | – | + |
| Ventral tegmental Area | – | – | + |
| Retrorubral field | – | – | + |
| Inferior colliculus | – | – | + |
| Anterior pretectal nucleus | – | – | + |
| **Diencephalon** | | | |
| Dorsomedial hypothalamic nucleus (DMH) in caudal hypothalamus | + | – | + |
| Ventrolateral preoptic nucleus (VLPO) in rostral hypothalamus | + | – | + (preoptic area) |
| Retrochiasmatic area and periventricular nucleus near suprachiasmatic nuclei | + (scattered neurons) | – | – |

*+ and – indicates presence or absence, respectively. *Phox2b mRNA has been detected in the locus coeruleus of adult rats by in situ hybridization (Fan et al., 2011).*

However, the reported improvement of respiratory parameters in two CCHS patients by ETO may also be explained by desogestrel activation of other areas of rodent brain (Loiseau et al., 2014; Joubert et al., 2016), including some expressing *Phox2b* in adulthood, as the C1 neurons (Stornetta et al., 2006), and catecholaminergic neurons in the ventrolateral medullary reticular nucleus (Joubert et al., 2016).

These data, along with *in vitro* and *in vivo* studies previously described, indicate that 3-KDG may have a more complex effect on general ventilation in CCHS patients acting on different respiratory networks.

## Other Pharmacological Targets

Recently, a child with CCHS due to heterozygous missense variant in exon 1, c.95A > T, showed an improvement in his daytime apneic episodes following treatment with carbamazepine (Schirwani et al., 2017), with no effects on sleep-related hypoventilation. Carbamazepine is mainly used as antiepileptic drug, because of its ability to reduce the iperexcitability of neurons by blocking voltage-gated sodium channels. It is also used as a mood stabilizer, especially to control maniac states, by exploiting its possible effects on noradrenaline in the brain. To explain the clinical improvement observed in the patient it has been hypothesized that carbamazepine may act by antagonizing A1 and A2 adenosine receptor, or by blocking noradrenaline receptors. Consistently, adenosine acts as a respiratory depressant, and adenosine antagonists are efficient respiratory stimulants, already used to treat neonatal apnea (Hascoet et al., 2000).

Another important class of possible drug targets is represented by ion channels. Neuronal cell excitability relies on their proper function, and in particular it has been reported that $K^+$ channels play a role in neuronal development and their activity modulates respiration *in vivo* (Malin and Nerbonne, 2002; Zavala-Tecuapetla et al., 2008; Lazarenko et al., 2010; Trapp et al., 2011; Torrecilla et al., 2013; Hawkins et al., 2015; Sobrinho et al., 2016).

Works by Bayliss' lab demonstrated that the intrinsic chemosensitivity of RTN neurons is due to two independent molecular pH sensors, namely TASK-2 (Wang et al., 2013;

Bayliss et al., 2015), an alkaline-sensitive $K^+$-channel of the two-pore domain (K2P) family, and GPR4 (Kumar et al., 2015), a proton activated receptor. Genetic deletion of both genes recapitulates the cardinal features of CCHS patients. On the other hand, these mice showed a residual chemosensitivity of RTN neurons and of the respiratory network, thus suggesting the activation of alternative/compensatory cellular and molecular mechanisms (Guyenet et al., 2016).

Recently (Mulkey et al., 2015), the KCNQ channels family has been reported to determine the chemoreceptor function of RTN, whereas the overexpression of the *KCNN3* gene induces abnormal respiratory responses to hypoxia (Mahida et al., 2014) and increased risk of sudden cardiac death (Bond et al., 2000; Mahida et al., 2014), thus indicating a possible use of drugs targeting them for ameliorating respiratory dysfunctions.

Several studies demonstrated that hypercapnia stimulates LC and C1 group neurons and has an excitatory effect on brainstem respiratory neurons (Viemari, 2008; Gargaglioni et al., 2010; Magalhães et al., 2018). These neurons express several chemosensitive $K^+$ channels (Gargaglioni et al., 2010, and references therein), among which the pH-sensitive Kir channels (D'Adamo et al., 2011), the leak-conductance two-pore domain TASK-1 channels (Bayliss et al., 2001), the large conductance $Ca^{2+}$-activated $K^+$ (BK) channels (Imber et al., 2018), the Kv channels and the acid-sensing ion channel (ASICs). The presence of multiple pH-sensitive ion channels, which may have different sensitivity to pH variation, can result in a fine modulation of neuronal response to hypercapnia, by means of a mechanism mainly based on inhibition of $K^+$ channels. The transient receptor potential (TRP) channels have also been implicated in the excitability of LC neurons in the presence of 8% $CO_2$ (Cui et al., 2011). They are $Ca^{+2}$ channels able to depolarize $CO_2$-sensitive neurons and their dysregulation could result in respiratory disorders.

The importance of ion channels as druggable targets in CCHS is supported by the commercial availability of drugs that potentiate or inhibit their function, already used in different clinical settings.

## CONCLUSION AND FUTURE PERSPECTIVES

The knowledge acquired so far on *PHOX2B* and its mutation, and its role in the etiogenesis of CCHS have strongly contributed to the improvement of the diagnosis and treatment of the patient, including the attempt to predict the severity of the disease by genotype-phenotype correlation analyses. The study of the impact of *PHOX2B* mutations on its functional activity has shed light on the identification of possible pharmacological targets to limit and/or counteract the toxic effect of mutant PHOX2B in order to improve respiratory symptoms. Among these, its target genes certainly represent the greatest challenge, as transcriptional dysregulation is among one of the pathogenetic mechanism involved.

The serendipitous observation about progestin improvement of respiratory parameters in two CCHS patients, and the evidence that younger patients show a residual cardiorespiratory response pave the way for new perspectives in the search for a therapeutic approach that can target the primary defect, or compensate for it by by-passing the primary defect.

However, the models available so far still limit our comprehension about PHOX2B role in the development of ANS and in the pathogenesis of CCHS. Immortalized cell lines may not recapitulate the physiology of a normal cell or do not have the genetic background of cells from human patients because of their genetic and metabolic defects. Although animal models are very useful to study autonomic circuits, connectivity, regulation, and related disorders, they have significant limitations due to the distinct physiologic mechanisms between rodents and humans. Postmortem tissue analysis is a great tool for studying cellular defects at the end of the disease, but shows some limitations for studying the molecular and biochemical pathophysiology of autonomic neurons. Moreover, in the case of CCHS, the availability of postmortem tissues is limited due to the rarity of the disease. Patient blood samples are also available to study ANS disorders, and recently in CCHS this has enabled to identify an increased oxidative stress in this rare disease (Degl'Innocenti et al., 2018). However, the use of peripheral tissues for studying disease mechanisms and developing treatment options in CCHS could be a problem, due to the fact that the disease does not manifest in blood tissue. The development of methods to efficiently obtain neurons from induced-pluripotent stem cells, obtained by reprogramming somatic cells of patients with a certain disease, is a challenging opportunity to investigate at the molecular level the developmental defect, in the same genomic background of the patient.

## The Disease in a Dish: The Induced Pluripotent Stem Cells (iPSCs) as a New Tool to Study CCHS Pathogenesis

Neurons obtained by differentiating human pluripotent stem cells (hPSCs), including embryonic stem or induced pluripotent stem cells (hESCs/hiPSCs), are a promising model for studying human neural development and disease (Avior et al., 2016; Pacitti et al., 2019), including rare pediatric disorders (Freel et al., 2020). Compared to hESCs, iPSCs can be obtained by reprogramming somatic cells (such as adult skin cells) from individual patients. Therefore, the iPSCs derived from fibroblasts of CCHS patients offer a new opportunity to obtain neuronal cells that carry the different mutations and to capture the entire patient's genetic profile, including all of the genetic modifiers that have important, yet largely unknown, roles in the pathology of CCHS (Bellin et al., 2012). This technology has previously been used to model familial dysautonomia (which, like CCHS, is a genetic disease related to ANS dysfunction) and has provided evidence that iPSCs can recapitulate disease-related phenotypes and are useful for studying molecular defects (Lee et al., 2009; Lee and Studer, 2011; Zeltner et al., 2016). Recently, it has been reported the generation of iPSC lines from two CCHS identical twins with *PHOX2B* PARM mutation (20/25 genotype). Both lines express pluripotency markers and can differentiate into the three germ

layers, but the possibility to use these iPSCs to recapitulate aspects of CCHS has not yet been fully investigated (Falik et al., 2020).

While significant progress has been made on the generation and utilization of CNS cell types from hPSCs, very few studies have been reported on the derivation of the neurons affected in CCHS [neural crest derived neurons and CNS neural lineages, such as central noradrenergic neurons (NANs), and hindbrain branchio motor (bMNs) and visceral motor neurons (vMNs)].

Reported methods of generating NANs and vMNs from pluripotent stem cells rely on the over-expression of *PHOX2B* (or alternatively *PHOX2A* for vMNs and bMNs) (Panman et al., 2011; Mong et al., 2014; De Santis et al., 2018).

Peripheral neurons can be generated from iPSCs using a multistage differentiation protocol that starts with the generation of neural crest (NC) precursors, as reported with the differentiation of NC cells from hESCs (Lee et al., 2007). This differentiation protocol has been modified and improved to efficiently obtain a higher number of pure PHOX2B$^{+}$ neurons (Chambers et al., 2009, 2013; Lee et al., 2010; Menendez et al., 2013; Huang et al., 2016; Oh et al., 2016; Zeltner et al., 2016; Frith et al., 2018; Kirino et al., 2018). ANS disorders, such as familial dysautonomia (Zeltner et al., 2016) and Hirschsprung's disease have been modeled by using hPSC (Workman et al., 2017). Interestingly, mutated enteric NC derived from hPSCs carrying a typical HD patients *PHOX2B* mutation showed a defect in migration and differentiation in intestinal organoids.

The setting up of *in vitro* protocols to obtain differentiated neurons relevant to CCHS is an ambitious task; one of the major challenges is in generating well-characterized, mature and functional cell types. Oh et al. (2016) use co-cultures of hPSCs-derived sympathetic neurons with cardiomyocytes to overcome this issue and to enhance the maturity of the neurons and improve functionality.

Despite the fact that the use of hPSC technology for CCHS modeling is still in its infancy its great potential to provide new tools for investigating CCHS pathogenesis and validating drugs to ameliorate CCHS symptoms is widely acknowledged in the scientific community.

## AUTHOR CONTRIBUTIONS

SD and RB contributed equally to the conceptualization and to the writing of the manuscript. SC contributed to writing and reviewing. DF revised and edited the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SYSTEMATIC REVIEW
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# Autonomic Characteristics of Sudden Unexpected Death in Epilepsy in Children—A Systematic Review of Studies and Their Relevance to the Management of Epilepsy in Rett Syndrome

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**Aim:** To systematically identify and critically appraise studies that investigate the autonomic characteristics of Sudden Unexpected Death in Epilepsy (SUDEP) in the pediatric population. We also wanted to explore how this information would be relevant to the management of epilepsy in patients with Rett Syndrome.

**Method:** Using PRISMA guidelines, a systematic review of PubMed, Scopus, Cochrane, PsycINFO, Embase, and Web of Science databases was performed to identify eligible studies. After extracting data from the included studies, a thematic analysis was undertaken to identify emerging themes. A quality appraisal was also done to assess the quality of the included studies.

**Results:** The systematic search revealed 41 records, and 15 full-text articles on the autonomic characteristics of SUDEP in children were included in the final analysis. Following thematic analysis, three themes were identified (I) modulation in sympathovagal tone, (II) pre- and post-ictal autonomic changes, and (III) other markers of autonomic dysregulation in children with epilepsy. Modulation in sympathovagal tone emerged as the theme with the highest frequency followed by pre- and post-ictal autonomic changes. While the themes provide additional insight into the management of epilepsy in the Rett Syndrome population, the quality of evidence concerning the autonomic characteristics of SUDEP in the pediatric population was low and underscores the importance of much needed research in this area.

**Conclusion:** The mechanism of SUDEP in the pediatric population is complex and involves an interplay between several components of the autonomic nervous system. While direct clinical inferences regarding pediatric SUDEP could not be made, the thematic analysis does suggest that in vulnerable populations such as Rett Syndrome,

where there is already a pervasive autonomic dysregulation, pro-active surveillance of the autonomic profile in this patient group would be useful to better manage epilepsy and reduce the SUDEP risk.

**Keywords: sudden unexpected death in epilepsy, epilepsy, autonomic dysregulation, Rett Syndrome, pediatric**

## INTRODUCTION

The management of epilepsy in children is clinically challenging and longitudinal follow-up studies have shown that death associated in young children with epilepsy is greater than the general population (1, 2). When a death occurs suddenly or is unexpected in children with epilepsy the term "Sudden Unexpected Death in Epilepsy" (SUDEP) has been ascribed. A more recent classification has expanded this definition (3) and encompasses the definitions of SUDEP + and takes into account if a comorbid disorder co-exists such as prolonged QT, however, the diagnosis of pure SUDEP is one that is based on exclusion. This disorder also shares substantial overlap with other disorders of sudden death, such as Sudden Infant Death Syndrome (SIDS) and Sudden Unexplained Death in Childhood (SUDC). Some overlapping features include evidence of hippocampal abnormalities, and association with disordered serotoninergic pathways. However, when SUDEP is compared to SIDS and SUDC, the defining feature is the clinical history of epilepsy and the event can occur at any age (4). Even though SUDEP is not dependent on age, some evidence suggests that the risk of SUDEP is about seven times greater in individuals with epilepsy age of onset between 0 and 15 years when compared to an age of onset ≥45 years (5, 6). Others have suggested that in those with childhood onset of epilepsy that does not fully subside, the lifetime SUDEP risk is 8% by 70 years of age (4, 7).

The event of SUDEP is probabilistic, and this is in part seen in the lack of consensus regarding the prevalence rates. While previous guidelines indicate an average incidence of 1.2/1,000 person-years for adults, and 0.2/1,000 person-years for children (8), some others have suggested that the incidence is far higher (9). An estimate of SUDEP incidence of 1.11/1,000 for children has been suggested (10). Further evidence has shown that even when adjusting for comorbid disorders, the risk of sudden death remains high in children with epilepsy (11) and underscores the importance for increased vigilance in this population. In the United Kingdom, deaths from epilepsy are increasing (12) and recent evidence from the North American SUDEP Registry has indicated that SUDEP can occur even in epilepsy that is relatively benign and treatment responsive (13). Despite this awareness, knowledge of SUDEP in the pediatric literature is relatively scarce, especially in neurodevelopment disorders of childhood that present with a clinical history of epilepsy.

Rett Syndrome (RTT) is a complex pediatric neurodevelopmental disorder characterized by comorbid symptoms and developmental delay. The frequency of epilepsy is varied in RTT. Data from the RTT Natural History study suggest a seizure prevalence ranging from 30 to 44%; however, the lifetime prevalence was up to 90% (14). Others have suggested prevalence rates of 82% (15), 76% (16), and 68.1% (17). Anti-seizure medications (ASMs) are also frequently used for treating epilepsy in RTT. In one study, 64% of patients were taking ASMs, and about 17% were reported not having seizures (18). Similarly, the age of the onset of epilepsy in RTT is variable ranging from 1 to 16 years age of onset (mean 5 years) (15). Other data suggest seizure frequency was about 11% in those under 4 years of age to a peak incidence of about 50% in the 16 to <20-year age group (14). Further, only about 8% of patients had onset after 20 years of age (14). In a study of 1,248 patients, the mean onset age of epilepsy was 4.68 ± 3.5 years of age (17). Despite these observations, there is no information in the literature concerning SUDEP in patients with RTT, especially in children. We do not know whether the trajectory of SUDEP changes over the periods of neurodevelopment in RTT, and neither do we know if tracking these changes would help in detecting early epileptic events that might lead to SUDEP.

Patients with RTT are at more risk of sudden death (19) and we know that the underlying epileptic seizures could potentiate brainstem vulnerability thereby increasing the risk of SUDEP in this patient group (20) especially in those with severe cardio-respiratory dysfunction. However, there are additional risk factors regarding SUDEP that should also be considered in the context of RTT. First, evidence (21) has shown that having three or more generalized tonic-clonic (GTC) seizures per year seems to be the highest weighted risk factor for SUDEP (9), followed by ≥13 of any type of seizure in the last 12 months (22). Second, polypharmacy is also an important risk factor, and data has shown that the SUDEP risk is increased in individuals taking ≥3 ASMs compared to monotherapy (22). Third, developmental delay is also suggested to be a risk factor (9, 23, 24). Fourth, children with complex epilepsy especially in those with associated neurodisability might also have an increased SUDEP risk (6, 9). These factors also feature on the SUDEP-7 risk inventory (9). When viewed together, these elements are also transferable risk factors for patients with RTT because this patient group has generalized seizures as a common seizure phenotype, have developmental delay and are usually prescribed with one or more ASM.

Given that the underlying autonomic impairment in RTT could help in identifying events that could lead to SUDEP (20), it would be prudent to explore studies relating to the autonomic

**Abbreviations:** ASMs, Anti-Seizure Medications; ECG, Electrocardiogram; EDA, Electrodermal Activity; EEG, Electroencephalograph; GTC, Generalized Tonic Clonic; HF, High Frequency; HRV, Heart Rate Variability; LF, Low Frequency; PGES, Post-ictal Generalized Electroencephalographic Suppression; QT, Q and T waves on ECG; RMSSD, Root Mean Square of Successive Differences; RTT, Rett Syndrome; R-R, Inter-Beat Interval; SIDS, Sudden Infant Death Syndrome; SUDC, Sudden Unexplained Death in Childhood; SUDEP, Sudden Unexpected Death in Epilepsy; SUDEP-7, Sudden Unexpected Death in Epilepsy Risk Inventory.

profile of children with SUDEP to see if we can identify patterns or hallmark features that might help to detect early changes that lead to SUDEP in RTT. The purpose of this study was (I) to systematically identify studies on the autonomic characteristics of SUDEP in children, (II) to appraise the identified studies to see whether we can recognize profiles or hallmark features of SUDEP in children, and (III) to use this knowledge to develop and propose any intervention that might enable the early detection of events that increase the risk of SUDEP in children with RTT. As the inherent nature of SUDEP is heterogenous, we were especially interested in whether the information extracted from the studies in children could aid in the development of biomarkers that would help to profile RTT patients deemed most at risk i.e., those on multiple ASMs, have a more severe breathing phenotype and have frequent seizures.

# METHODS

To perform the systematic review, two authors (JS and EL) independently followed the PRISMA guidelines (25) to search the PubMed, Scopus, Cochrane, PsycINFO, Embase, and Web of Science databases during October 2020 in a blinded manner. To ensure the search was expansive and captured the relevant search terms, the truncation symbol (*) was used.

## Search Terms

The following search terms were used:
(Sudden Unexpected Death in Epilepsy OR SUDEP) AND (autonomic variables OR autonomic parameters) AND (child* OR pediatric).

## Population Characteristics

Databases were searched for records that looked for studies in children that mentioned SUDEP.

## Intervention

All studies that mentioned or reported autonomic characteristics or parameters were included.

## Eligibility Criteria

The following inclusion and exclusion criteria were used:
Inclusion Criteria

- ➢ Full-text records in peer-reviewed academic/scientific journals
- ➢ Studies or investigations done in humans and available electronically

Exclusion Criteria

- ➢ Studies not available electronically and not available in English
- ➢ Reviews, case reports, and preprints.

## Critical Appraisal of Eligible Articles

The quality of the eligible articles was determined using the appraisal checklist developed previously (26) and has been used in systematic reviews of RTT syndrome (20, 27). In the present study, the procedure to critically appraise the 15 articles against the 11 criteria was followed as described in our previous evidence synthesis (20).

## Data Extraction and Analyses

The methods of data extraction and analysis was performed as previously described (20). To minimize bias in the search process, data extraction and analysis, we used the following strategies:

(I) Two authors (JS and EL) blindly and independently performed the systematic review. The eligible articles were based on a consensus agreement between JS and EL. If agreement could not be reached, then the senior author (PS) was consulted.
(II) The first author (JS) performed the manual coding as previously described (20) to identify preliminary themes. The second author (EL) then independently reviewed these themes and then afterwards, a consensus was reached between JS and EL on the themes that emerged. Lastly, the themes were reviewed by the senior author (PS), and the final themes were based on an agreement between all three authors.

The frequencies of the themes were presented using Microsoft Excel software 2016.

# RESULTS

The systematic search of the databases revealed 41 records, and after duplicates were removed 28 articles remained (**Figure 1**). The title and abstract of these articles were screened, and five articles were excluded. Twenty three full-text articles were then assessed against the eligibility criteria, and a further eight articles were removed. The remaining 15 full text articles were included in the analysis, and the autonomic characteristics from each of these articles are presented in **Table 1**.

## Study Characteristics

The studies were expansive in terms of evaluating different aspects of autonomic dysregulation in children with epilepsy. This included assessment of heart rate variability (HRV) parameters before and after GTC seizure onset with and without Post-ictal Generalized Electroencephalographic Suppression (PGES) (28). Indices of HRV were also used to profile focal and generalized seizures in children (30). Another study explored HRV parameters in intractable epilepsy (32) or children with epilepsy during sleep (35). These studies were useful to see if patterns in the sympathovagal balance could be identified to assist in the development of potential prognostic markers for SUDEP. The developmental trajectory of PGES alongside the amplitude of electrodermal activity (EDA) across different age ranges was also explored (36). In some studies, specific aspects of HRV indices were assessed in children with refractory epilepsy and compared to those where the epilepsy was better controlled (37). Properties of the R-R interval during the pre-ictal period were also assessed (38). The relationship between PGES and peri-ictal tachycardia and hypoxemia in children with epilepsy was examined. This study was useful because the

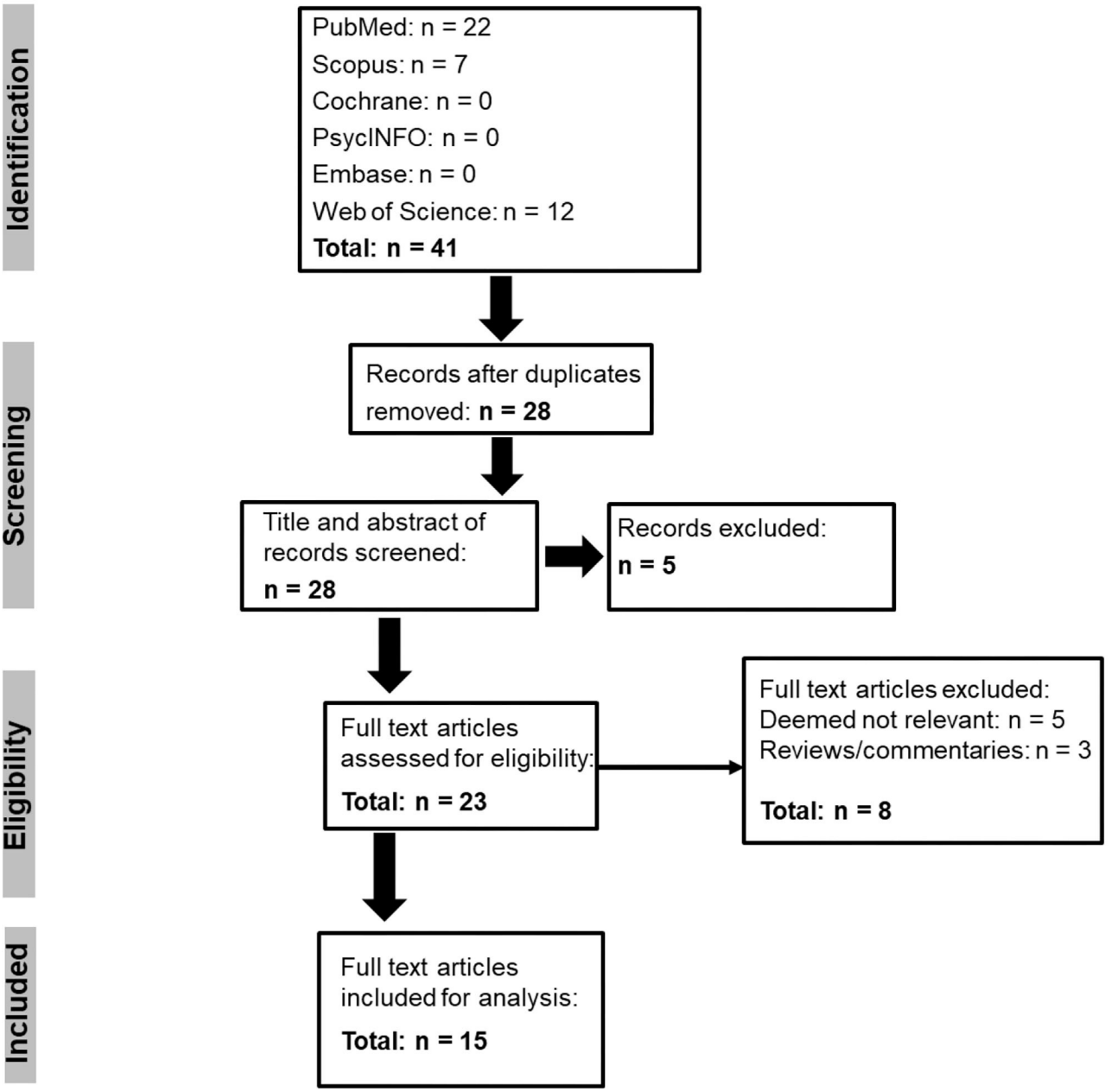


**FIGURE 1 |** PRISMA flow-diagram.

observations could also be correlated with SUDEP-7 inventory scores (29).

The interplay between different components of the autonomic nervous system (ANS) was also assessed in generalized subclinical and seizures of temporal origin (39). This aspect was further explored in intractable and well-controlled epilepsy (31). The autonomic characteristics of patients on ASMs and how this compares to patients without treatment were also investigated (40). Some other studies provided a broader overview of the sympathovagal profile in children with epilepsy (41–44).

## Thematic Analysis

Based on a consensus agreement between all the authors, three themes emerged from the eligible studies evaluating SUDEP in the pediatric population. The frequency of these themes is shown in **Figure 2** and are named as:

Theme 1: Modulation in sympathovagal tone
Theme 2: Pre- and post-ictal autonomic changes
Theme 3: Other markers of autonomic dysregulation in children with epilepsy.

**TABLE 1 |** Summary of eligible studies relating to the autonomic characteristics of SUDEP.

| Source | Demographics | Clinical characteristics | Assessment methods | Relevant autonomic information |
|---|---|---|---|---|
| Okanari et al. (28) | • Thirty five children aged between 3 and 18 years who had GCS.<br>• Seventeen age-matched controls. | • In the 35 children, 74 instances of GCS were identified.<br>• Of the 74 GCS, 36 of these also showed PGES and 38 GCS were without PGES. | • Video EEG and ECG (1 lead monitoring).<br>• Pre-, inter-, and post-ictal measurements of HRV parameters including LF, HF, LF/HF, and RMSSD. | • The pre-ictal autonomic parameters LF and HF in children with 36 GCS+PGES was significantly greater ($P < 0.01$) when compared to 38 GCS without PGES.<br>• Post-ictal RMSSD was higher in the GCS+PGES group than the GCS-PGES group ($P < 0.01$) and the pre to post-ictal change in RMSSD was lower in children with GCS and PGES than those that had GCS without PGES ($P = 0.035$).<br>• No changes in inter-ictal HRV parameters were observed among the GCS and the control group.<br>• Measurement of HRV parameters could be useful to identify those subsets of high-risk children such as those with abnormal GCS+PGES changes that might lead to SUDEP. |
| Pernice et al. (30) | Thirty seven children ($n = 20$ males and $n = 17$ females) aged 6.27 ± 5.1 years of age. | • The children had either focal ($n = 23$) or generalized ($n = 14$) seizures.<br>• Patients were treated with ASMs based on their diagnosis. | Nine HRV parameters were measured in time, frequency, and entropy domains. | • HRV analysis was able to discriminate between focal and generalized seizures.<br>• During the post-ictal phase, children with focal seizures had elevated heart rate, depressed HRV and increases in LF and the LF/HF ratio.<br>• In comparison to children with focal seizures, seizures in children with generalized epilepsy were characterized by increases in the normalized LF, LF/HF ratio, and a lower mean RR interval and RMSDD before the seizure.<br>• Monitoring of HRV can be useful in identifying shifts in the sympathovagal balance reflected by changes in focal seizures or during periods of generalized seizures.<br>• A dominant sympathetic profile and vagal withdrawal are thought to be characteristic in children with generalized seizures during the pre-ictal period. |
| Yang et al. (32) | • Fifty one patients ($n = 34$ males and $n = 17$ females) aged between 6 and 38 years of age.<br>• Fifty age and gender matched controls. | • All patients had refractory epilepsy and were treated with either one or more ASMs.<br>• The mean (SD) number of seizures per month were 103.1 (174.4).<br>• Of the 51 patients, 38 were on polytherapy and 13 were on monotherapy. | Time, frequency and non-linear domain HRV parameters using 24-h ECG | • The findings showed that patients with refractory epilepsy had significantly lower time, frequency, and non-linear domain parameters than healthy controls.<br>• The difference in the HRV parameters between the epilepsy and control groups was the highest in the early morning.<br>• Altered sympathovagal imbalance as reflected by impaired HRV parameters might be useful for the development of prognostic markers of SUDEP |
| Sivakumar et al. (35) | • The generalized epilepsy group consisted of 91 subjects with a mean (SD) age: 10.5 (5.0) years.<br>• The comparator group was a control group of 25 subjects with a mean (SD) age: 7.5 (6.4) years | • All subjects had a diagnosis of epilepsy.<br>• Subjects were on a ketogenic diet and were taking ASMs.<br>• During the overnight period, subjects were asked not to take their medications. | • Retrospective review of medical records.<br>• Measurement of HRV, ECG and EEG waveforms.<br>• ECG traces were explored during 30 min of stage 2 sleep. | • In the absence of seizures, there was increased RSA and lower heart rate in children with epilepsy during sleep.<br>• These findings suggest an increased vagal tone in children with generalized seizures.<br>• It was proposed that an increase in parasympathetic tone could precede the onset of epilepsy in children. |
| Sarkis et al. (36) | • Twenty patients were included.<br>• Seven were in the age range of 11–17 years (younger age group) and 13 were adult patients (18–67 years). | • All patients that had analyses wore an EDA sensor.<br>• MRI lesions were noted in some of the patients.<br>• Mean (range) duration of epilepsy was 11.1 years (1–51)<br>• Focal seizures accounted for 80% of the epilepsy type.<br>• Number (mean [range]) of ASMs was 2.5 (1–5) | • Use of an EDA wrist sensor.<br>• EEG and ECG measurements. | • The study showed that there was a strong correlation between the duration of PGES and age ($P = 0.004$).<br>• When the first GTC seizure was compared between the adult and the younger age groups, it showed that younger patients had a higher EDA amplitude than the adult group (11.80 μS ± 6.94 vs.5.19 ± 3.40 μS $P = 0.03$), suggesting a greater degree of sympathetic activation.<br>• The mean % change in HF power was also higher in the younger age group in comparison to adults (–97.78 ± 6.0 vs.–72.7 ± 15.0, $P = 0.0016$) reflecting increased vagal suppression.<br>• Following a GTC seizure and controlling for PGES duration, patients of a younger age are suggested to have enhanced sympathetic activation and vagal suppression. |

*(Continued)*

**TABLE 1 |** Continued

| Source | Demographics | Clinical characteristics | Assessment methods | Relevant autonomic information |
|---|---|---|---|---|
| Kolsal et al. (37) | • Group 1 (refractory epilepsy; $n$ = 20): Mean age ± SD 9.55 years ± 5.02<br>• Group 2 (controlled epilepsy; $n$ = 20): Mean age ± SD 10.1 years ± 4.18<br>• Group 3 (healthy controls; $n$ = 20): Mean age ± SD 10.35 years ± 4.39 | • Children with refractory epilepsy were using three or more ASMs.<br>• All patients were assessed by a Pediatric Cardiologist. | • HRV measurements using Holter and 12-lead ECG.<br>• Video EEG<br>• Brain MRI evaluation | • Children with epilepsy have abnormal QTcD and have depressed HRV.<br>• The time domain autonomic parameters RMSSD and SDNN in patients with treatment resistant epilepsy was also lower than the other two groups.<br>• A disruption in the vagal tone reflected by changes in the LF/HF ratio before and during seizures suggests that the sympathovagal balance is considerably stressed in children with epilepsy, and the sympathetic component is thought to dominate before seizure onset. |
| Jansen et al. (38) | • Seizures were monitored from patients aged 9.2 years<br>• Patients were selected from a group of 35 patients | • EEGs of 80 seizures were analyzed pre- and post- seizure onset.<br>• Seizures were of focal onset ($n$ = 40) and of generalized onset ($n$ = 40) | • Video EEG<br>• HRV time and frequency domain parameters. | • The R-R interval was useful in detecting pre-ictal heart rate changes in 70% of focal seizures.<br>• In focal seizures, the pattern of mean R-R was different before the seizure onset when compared to after seizure onset, and the duration of pre-ictal HRV to seizure onset is short.<br>• It was proposed that change in heart rate might be useful in detecting aberrant changes that manifest prior to the onset of temporal and frontal lobe seizures in children. |
| Moseley et al. (29) | • Thirty seven patients (male $n$ = 13; female $n$ = 24)<br>• Age at admission was: 10.2 ± 4.8 years.<br>• Age at seizure onset was 5.3 ± 4.5 years | • Children were included if they had one documented focal or primary/secondary GTC seizure.<br>• In 40.5% (15) children there was developmental delay. | • EEG alongside ECG and pulse oximetry measurements.<br>• SUDEP-7 inventory score. | • Only GTC seizures were characterized with PGES.<br>• PGES was shown to account for about 16% (27/168) of the seizures in 32% (12/37) of children, and was significantly associated with peri-ictal tachycardia ($P$ = 0.019) and hypoxemia ($P$ = 0.005).<br>• Mean duration of PGES was 35.1 ± 19.6 s, and in 10 children the PGES was deemed to be prolonged (≥30 s).<br>• Children with PGES also had higher SUDEP-7 inventory scores than children without PGES (4.2 ± 1.3 vs. 2.8 ± 1.4, $P$ = 0.007). This might suggest that children with PGES during a GTC seizure could be at higher risk of SUDEP. |
| Brotherstone and McLellan (39) | • Eleven patients were included with an age range of 3 years 1 month to 60 years 3 months<br>• Six patients were adults (male $n$ = 3; female $n$ = 3) and five patients were pediatric (male $n$ = 4; female $n$ = 1). | • From the 11 patients 33 sub-clinical seizures were recorded with a mean duration of 191.1 s ± 136.4 (range: 63–340 s).<br>• The 33 seizures were classified as being generalized ($n$ = 19), right temporal lobe ($n$ = 9), and left temporal lobe ($n$ = 5) | • Prospective measurement of video EEG, ECG, and oxygen saturation recordings.<br>• NeuroScope analysis | • Generalized sub-clinical seizures showed larger increases in cardiac vagal tone and less change in HRV when compared to temporal lobe sub-clinical seizures.<br>• The findings showed that during generalized sub-clinical seizures there is an elevated parasympathetic activity, however, seizures originating from the temporal lobe showed lower parasympathetic activity.<br>• During sub-clinical generalized seizures there is autonomic dysregulation characterized by changes in the parasympathetic component. |
| Mukherjee et al. (31) | • Group 1 (intractable epilepsy; $n$ = 31 [male $n$ = 22; female $n$ = 9]): Age 22.11 ± 10.18<br>• Group 2 (well-controlled epilepsy; $n$ = 30 [male $n$ = 18; female $n$ = 12]): Age 19.13 ± 8.72 | • All patients were confirmed as having intractable or well-controlled epilepsy.<br>• In Group 1, 25 subjects were on two ASMs while five were treated with three ASMs.<br>• In Group 2, 26 subjects were on one ASM, relatively stable and four were on two ASMs. | • Range of tests for autonomic function including the deep breathing test, Valsalva maneuver, hand grip test, cold pressor test, and head up-tilt test.<br>• Cardiovascular tone (respiration and ECG waveform, and time domain analyses).<br>• Neuropsychological assessment of anxiety using clinician rated questionnaires.<br>• Autonomic symptom score consisting of seven autonomic indices. | • Patients with intractable epilepsy (Group 1) had higher LF and lower HF values than the well-controlled group.<br>• Group 1 was noted to have a higher autonomic dysregulation as evidenced by a higher sympathetic tone, lower parasympathetic tone, and lower parasympathetic reactivity.<br>• It was indicated that patients with intractable epilepsy have a different and more severe autonomic profile than those with well-managed epilepsy, and that these patients could be at higher risk from SUDEP. |

*(Continued)*

**TABLE 1 |** Continued

| Source | Demographics | Clinical characteristics | Assessment methods | Relevant autonomic information |
|---|---|---|---|---|
| Hallioglu et al. (40) | • Group 1 (epilepsy patients on treatment; $n = 78$): Mean age ± SD 7.2 years ± 4.3<br>• Group 2 (epilepsy patients without treatment; $n = 14$): Mean age ± SD 8.2 years ± 2.7<br>• Group 3 (healthy controls; $n = 83$): Mean age ± SD 8.1 years ± 3.4 | • Of the 92 patients with epilepsy, 14 had a new diagnosis and did not receive ASM.<br>• Of the 78 patients using ASMs 33 used valproic acid. 19 used oxcarbazepine, 11 phenobarbital, 10 were on combined treatments and 5 received other drugs. | • ECGs<br>• Measurement of time and frequency domain HRV indices. | • The findings showed that the HRV and time domain measures (RMSSD, SDNN and HRV triangular index) were decreased in epilepsy patient regardless whether they were on ASM.<br>• However, patients not on any ASMs were said to have a lower parasympathetic activity as indicated by lower HF values and an increased LF/HF ratio.<br>• The parasympathetic autonomic profile is more suppressed in patients not on ASM. |
| Harnod et al. (41) | • Thirty children (15 males and 15 females) with a mean age 10.9 ± 0.6 years<br>• The control group had 30 individuals (15 males and 15 females) with a mean age of 10.6 ± 0.6 years | • All children with epilepsy had recurrent seizures and were on ASMs<br>• Duration of epilepsy was 6.1 years ± 0.7 | ECGs to characterize and assess frequency domain analysis of HRV | • The epilepsy group had lower frequency domain indices (R-R, LF, and HF) when compared to the control group.<br>• It was proposed that in children with intractable epilepsy there is lower HRV due to a decrease in the parasympathetic component. |
| El-Sayed et al. (42) | • Twenty-five young people (13 males and 12 females) with a mean age 10.36 years ± 4.0<br>• The control group consisted of 50 individuals (26 males and 24 females) with a mean age of 11.0 years ± 3.5 | • Patients had both partial and generalized epilepsy.<br>• Generalized seizures were present in 10 patients and 15 had focal related epilepsy.<br>• Patients were on monotherapy (13 on valproate or 12 on carbamazepine). | • Clinical scoring of five autonomic function test including resting heart rate, heart rate response to deep breathing, Valsalva maneuver, 30:15 ratio heart rate response to standing and blood pressure response to standing.<br>• Time domain HRV measurements. | • SDNN was found to be lower across all age groups.<br>• All patients with uncontrolled epilepsy had abnormal autonomic dysregulation (83% had moderate autonomic and one [17%] had mild autonomic dysregulation).<br>• Seizure type and type of ASM had no discernable effect on the outcome of clinical scoring of autonomic tests.<br>• Based on clinical autonomic scoring, patients with uncontrolled epilepsy had a higher degree of autonomic dysregulation. |
| Ferri et al. (43) | • Eleven children (5 males and 6 females) with a mean age ± SD of 11.5 years ± 3.65<br>• The control group consisted of 11 (5 males and 6 females) individuals aged (mean ± SD) 12.9 years ± 2.72 | • All children had partial epilepsy and were treated with one or more ASMs.<br>• Diagnosis was based on EEG and neuroimaging. | • Sleep EEG<br>• Time and frequency domain HRV measurements | • The study showed that in the patients with epilepsy during sleep had lower time and frequency domain HRV values.<br>• The sympathovagal balance (LF/HF) ratio was higher in patients with epilepsy especially during sleep when compared to the control group.<br>• During REM sleep there can be altered autonomic patterns in children with partial epilepsy. |
| Yang et al. (44) | • Thirty children (21 males and 9 females) with a mean age ± SD of 6.0 years ± 1.3<br>• The control group consisted of 30 age and gender matched healthy individuals without a history of neurodevelopmental disorders. | • Of the 30 children, 22 also had neurodevelopmental disorders such as cerebral palsy and developmental delay.<br>• The profile of seizures included 18 cases of GTC seizures, 10 with partial seizure and 2 with absence seizures. | ECG measurements and frequency domain HRV analysis. | • Mean age of seizure onset was 26.6 months and the mean length of seizure disorder was 4.6 years.<br>• Children with epilepsy were noted to have an abnormal sympathovagal imbalance. |

*ASMs, Anti-Seizure Medications; EDA, Electrodermal Activity; EEG, Electroencephalography; ECG, Electrocardiogram; GCS, Generalized Convulsive Seizures; GTC, Generalized Tonic Clonic; HF, High Frequency; HRV, Heart Rate Variability; LF, Low Frequency; MRI, Magnetic Resonance Imaging; PGES, Post-ictal Generalized Electroencephalographic Suppression; QTcD, QTc dispersion; RMSDD, Root Mean Square of Successive Differences; R-R, Inter-beat Interval; RSA, Respiratory Sinus Arrhythmia; SD, Standard Deviation; SDNN, Standard Deviation of all NN Intervals; SUDEP, Sudden Unexpected Death in Epilepsy.*

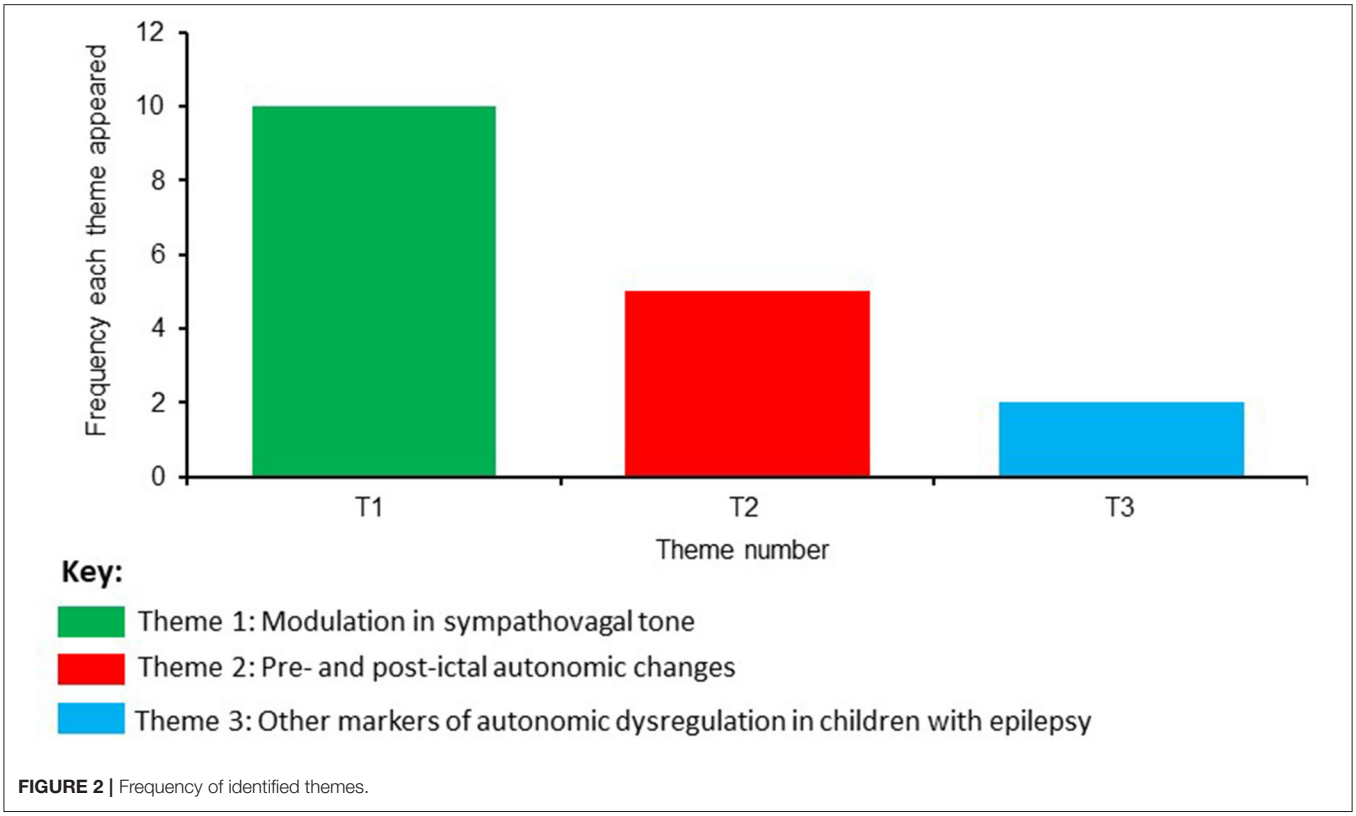


**FIGURE 2 |** Frequency of identified themes.

The most frequent theme that emerged was regarding the modulation in sympathovagal tone, followed by autonomic changes before and after seizure onset (pre- and post- ictal autonomic changes). The theme with the lowest frequency was related to other markers of autonomic dysregulation. The main results from these themes will be described in the next section:

### Theme 1: Modulation in Sympathovagal Tone

Changes to the sympathovagal tone emerged from studies that investigated the autonomic characteristics of epilepsy in children to assess whether there is an underlying autonomic dysregulation. One important aspect that arose from this theme was the detection of sympathovagal changes before seizure onset and how this might alter based on seizure localization. For example, it was shown that shortly after focal seizure onset, there is tachycardia, decreased HRV, and increased sympathovagal imbalance as indicated by an increased Low Frequency/High Frequency (LF/HF) power (30). However, when assessing the autonomic phenotype of generalized seizures, it was found that children had tachycardia, decreased Root Mean Square of Successive Differences (RMSSD) and increased LF/HF power before seizure onset. These observations provide evidence for a differential diagnosis of seizure phenotype from the perspective of autonomic indices between focal and generalized seizures in children. In particular, it suggests that during the pre-ictal period, there is a vagal decline characterized by a sympathovagal shift toward the sympathetic component in children with epilepsy. From a clinical viewpoint, this is relevant because (I) peri-ictal reductions in vagal tone can increase cardiac dysfunction leading to short term changes in tachycardia and fibrillation (38, 45) and (II) a decrease in RMSSD has been shown to be associated with higher total scores on the SUDEP-7 risk inventory (9, 46).

The sympathovagal decline also seems to be a characteristic phenotype in children with refractory epilepsy. These children are also suggested to have lower time, frequency and non-linear domain HRV parameters when compared to age and gender-matched controls (32), and it was suggested that the decline in vagal tone is due to decreases in both the sympathetic and parasympathetic components of the ANS. The amplitude of this change was also demonstrated to increase at night and peaked in early morning (32). This aligns with other data in children with generalized epilepsy that show increased respiratory sinus arrhythmia and lower heart rate during sleep than control subjects (35). This study also suggested that an elevated parasympathetic tone is an autonomic characteristic that precedes the onset of seizures in children. Another study had also demonstrated decreased time and frequency domain HRV parameters during sleep in children with epilepsy (43).

Modulation in sympathovagal tone can also be useful in pinpointing features between generalized sub-clinical seizures to those that originate from the temporal lobe. In another study, generalized subclinical seizures were also shown to present with increased parasympathetic activity when compared to seizures originating from the temporal lobe, and could indicate an increased autonomic vulnerability in children with generalized

sub-clinical seizures (39). When looking more specifically at epilepsy that is intractable and comparing it with control subjects, there is evidence to suggest a higher autonomic dysregulation in children with intractable epilepsy (31), which could be driven by a decrease in the parasympathetic component (41), and reduction in time domain HRV parameters (42). In summary, time-domain and HRV parameters are reduced in children with epilepsy, and those not on any ASMs showed a trend toward a more suppressed parasympathetic autonomic profile (40).

### Theme 2: Pre- and Post-ictal Autonomic Changes

Following the theme concerning changes to the sympathovagal tone, the second most frequent theme that emerged was related to pre- and post-ictal autonomic changes. Post-ictal generalized EEG suppression (PGES) occurs after a seizure, and it has been suggested that PGES may be a reflection of brainstem shutdown and a failure of arousal mechanisms (28). This adds weight to the hypothesis that PGES could be a potential marker for SUDEP, especially in instances where its duration is longer than 50 s (47), however, others have indicated that the duration of PGES does not seem to be a risk factor for SUDEP (48, 49). Notwithstanding this inconsistency, in children with generalized seizures, pre-ictal autonomic parameters (LF and HF) were found to be higher in those children with Generalized Convulsive Seizures (GCS) and PGES than without PGES (28). This suggests that children with GCS and PGES together have a more disturbed autonomic dysregulation and potentially higher risk of SUDEP. Post-ictal RMSSD values were also higher in children with GCS and PGES. Higher post-ictal RMSDD in children with GCS with PGES could be a pre-cursor for events leading to SUDEP. There is limited data on the neurodevelopmental risk of SUDEP. Some evidence has shown that the duration of PGES is associated with age with adults having a longer duration of PGES than children and that sympathetic activity during the pre-ictal period correlates with the duration of PGES (36).

Assessment of the R-R interval can also be useful in detecting pre-ictal heart rate changes in temporal and frontal lobe seizures. In seizures of temporal or focal localization, the pattern of heart rate changes was shown to be different pre and post-seizure onset (38). However, this pattern was not found in children with generalized seizures. Generalized seizures in children are noticeable due to PGES (29). In this study, PGES was reported in about 16% of the seizures in 32% of children. The average duration of PGES was 35.1 $\pm$ 19.6 s, and in 10 children, the duration of PGES was $\geq$30 s. Peri-ictal tachycardia was the most frequent autonomic characteristic noted in about 40% of seizures. While there was no significant association between peri-ictal tachycardia and the duration of PGES, the presence of peri-ictal tachycardia did show a significant association with PGES ($P = 0.019$). Similarly, PGES was shown to be associated with peri-ictal hypoxemia ($P = 0.005$) and there was also a trend toward peri-ictal hypoxemia and the duration of PGES ($P = 0.054$). Children with PGES were also shown to have higher scores on the SUDEP-7 inventory ($P = 0.007$). When viewed together, the findings from this study demonstrate that (I) in children with PGES there is an association with the presence of peri-ictal tachycardia and hypoxemia and (II) following a generalized seizure, the occurrence of PGES could potentially increase the risk of SUDEP in these children.

### Theme 3: Other Markers of Autonomic Dysregulation in Children With Epilepsy

This theme incorporated other potential markers of autonomic dysregulation in children with epilepsy. When GTC seizures were compared between adults and younger patients, children were shown to have higher EDA values (36). Since EDA reflects changes in sympathetic activation (50, 51), this finding suggests that when controlling for the length of PGES, children with GTC seizures have higher sympathetic activation than adults. When looking at cardiac parameters, children with refractory epilepsy were found to have prolonged QTc dispersion (QTcD) (37). This finding is important because a previous 2-year review of seizures in a pediatric unit showed ictal arrhythmias were present in 40% of patients (34) and might suggest that patients with epilepsy are more predisposed to increases in QTcD.

## Quality Appraisal of the Eligible Articles

Each of the 15 included articles in the study was assessed against 11 eligibility criteria (**Table 2**). The majority of the studies included children, but two had a mixed population (36, 39). None of the studies provided a formal sample size estimate to determine whether the studies were sufficiently powered; however, some studies did acknowledge this limitation. Studies also used a variety of methods ranging from EEG, HRV and specific tests for autonomic and cardiac function. While the studies do provide important information regarding the autonomic characteristics of HRV and its potential as a prognostic biomarker in SUDEP, none of the studies included data specifically on SUDEP in children, and only one study had correlated the findings related to PGES to the SUDEP-7 inventory (29). This finding is not unexpected as there is very limited data regarding SUDEP in children. While the topic of SUDEP has been discussed previously (5, 33), there is very little empirical data regarding pediatric SUDEP. In the present evidence synthesis, the quality appraisal suggests that none of the identified studies can provide direct, clinically meaningful inferences regarding SUDEP in children. Despite this limitation, the studies were valuable in providing information on autonomic characteristics that would be useful for managing risk in pediatric SUDEP. The SUDEP-7 inventory is a surrogate measure of SUDEP risk and includes two potential biomarkers of SUDEP risk—RMSSD (46) and PGES (29, 36), and the thematic analysis showed that RMSDD and PGES are factors that should be considered for managing risk in pediatric SUDEP. Furthermore, epilepsy can itself cause reductions in HRV (37) and respiratory sinus arrhythmia together with mean heart rate can identify children with epilepsy before the clinical signs become apparent (35).

In summary, the quality appraisal shows that while no direct clinical comparison can be made from the information provided in the articles to pediatric SUDEP, the thematic analysis does suggest that the autonomic characteristics of the studies would be useful for managing risk is pediatric epilepsy. This aligns with a recent systematic review of SUDEP in children that suggested even though the data relating to the causes of pediatric SUDEP is

**TABLE 2 |** Quality assessment of reviewed studies on autonomic characteristics in children with SUDEP.

| Study | Criteria* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1. Was the sample characteristic of the specific population?** | **2. Were patients recruited in an appropriate way?** | **3. Was the sample size sufficient to power the study?** | **4. Were the study participants described in detail and fosters comparison with other relevant studies?** | **5. Was the data analysis undertaken with adequate description of the identified sample?** | **6. Were objective and standard criteria used for the measurements?** | **7. Were the assessment and measurement methods used reliably?** | **8. Were the statistical analyses used appropriate?** | **9. Were relevant confounding factors described and accounted for?** | **10. If sub-populations were identified, were they done according to objective criteria?** | **11. Was there a conflict of interest?** |
| Okanari et al. (28) | No—children with PGES and healthy age matched controls were included but cohort did not specifically examine SUDEP | N/A—review of retrospective data collection | Unclear (the authors mention that the study was performed in a single tertiary care center and the cohort did not include information from a population based sample) | Yes | Yes | Yes, EEG and HRV measurements were used. | Yes | Yes, to compare autonomic characteristics with and without PGES | Yes, sleep stages that could influence HRV was mentioned | N/A | No |
| Pernice et al. (30) | No—cohort did not specifically assess SUDEP | Yes | Unclear—no information about sample size estimates were provided | Yes | Yes | Yes | Yes | Yes, nine indices of HRV were analyzed. | Yes | N/A | Unclear—no conflict of interest statement was provided |
| Yang et al. (32) | No—the study did not specifically include SUDEP | Yes—although the study included a mixed age range from 6 to 38 years | Unclear—although it was mentioned more sampling and accurate data would be needed for further SUDEP studies | Yes | Yes | Yes, ECG data was analyzed using Kubios software and cosinor fit method was used to assess the circadian HRV rhythm | Yes | Yes, time, frequency and non-linear domain indices were analyzed | Yes, the different phenotypes of epilepsy in the patient population was mentioned along with the ASM of patients | N/A | No |
| Sivakumar et al. (35) | No—data from SUDEP was not specifically assessed | N/A—retrospective data collection from a single pediatric epilepsy unit | Unclear—the need for further subjects was indicated including those with focal epilepsy | Yes | Yes | Yes | Yes | Yes, analyses of ECG and EEG data | Yes | N/A | No |
| Sarkis et al. (36) | No—although the paper stated implications for SUDEP, the study cohort did not include data from SUDEP | Yes—study population was mixed (13 were adult patients aged 18–67 years) | No—the authors mention that the study was limited due to the small sample size (only subjects with GTC seizures were analyzed) | Yes | Yes | Yes | Yes | Yes, EEG, HRV and EDA analyses | Yes, age and seizure capture were mentioned | N/A | Yes, a disclosure statement for the authors was provided |
| Kolsal et al. (37) | No—the cohort did not assess SUDEP | Yes | Unclear—no indication of sample size estimates was mentioned | Yes | Yes | Yes | Yes | Yes, HRV analyses | Yes | Yes, alongside refractory epilepsy a well-controlled epilepsy group was also included | No |
| Jansen et al. (38) | No—SUDEP was not assessed | Yes | Unclear, although the limitations of the small sample were considered when comparing the difference between temporal lobe (mesial and lateral) seizures | Yes | Yes | Yes | Yes | Yes, EEG and ECG analyses | Yes | N/A | Unclear as no conflict of interest statement was provided |

*(Continued)*

**TABLE 2** | Continued

| Study | Criteria* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **1. Was the sample characteristic of the specific population?** | **2. Were patients recruited in an appropriate way?** | **3. Was the sample size sufficient to power the study?** | **4. Were the study participants described in detail and fosters comparison with other relevant studies?** | **5. Was the data analysis undertaken with adequate description of the identified sample?** | **6. Were objective and standard criteria used for the measurements?** | **7. Were the assessment and measurement methods used reliably?** | **8. Were the statistical analyses used appropriate?** | **9. Were relevant confounding factors described and accounted for?** | **10. If sub-populations were identified, were they done according to objective criteria?** | **11. Was there a conflict of interest?** |
| Moseley et al. (29) | No—children with PGES had higher SUDEP-7 inventory score but the study cohort did not include information specifically related to SUDEP | Yes | Unclear—the study analyses took account the small sample size and fewer patients with PGES | Yes | Yes | Yes | Yes, including a surrogate measure of SUDEP (SUDEP-7 inventory) | Yes | Yes, the authors described this in detail | Yes | Unclear as not conflict of interest statement was provided |
| Brotherstone and McLellan (39) | No—although a SUDEP mechanism was proposed the study did not formally assess SUDEP | Yes—study was a mixed population (six patients were adults) | N/A—study was a pilot study | Yes | Yes | Yes | Yes, the use of Neuroscope and BioSignal HRV | Yes | Yes | N/A | No |
| Mukherjee et al. (31) | No—sample did not formally assess SUDEP | Yes | No—the study was not sufficiently powered to explore gender and age | Yes | Yes | Yes, a range of tests for autonomic function were performed | Yes | Yes and the small sample size was factored into the analyses | Yes | N/A | Unclear—as no statement was given |
| Hallioglu et al. (40) | No—the cohort did not assess SUDEP | Yes | Unclear—although it was mentioned that the sample size was too small when groups were split based on their ASMs | Yes | Yes | Yes | Yes, time and frequency domain measure of HRV | Yes | Yes | Yes, were divided into sub-groups based on ASMs | Unclear—no statement was provided |
| Harnod et al. (41) | No—no assessment of subjects with SUDEP | Yes | Unclear—no sample size statement provided | Yes | Yes | Yes | Yes, frequency domain measurements of HRV | Yes | Yes—patient characteristics (such as the exclusion of those with partial or controlled seizures) | N/A | Unclear—no statement provided |
| El-Sayed et al. (42) | No—cohorts did not include information on SUDEP | Yes | Unclear—although the small number of patients was acknowledged in the study. | Yes | Yes | Yes, five tests for cardiac autonomic function | Yes | Yes | Yes | N/A | Unclear—no statement was provided |
| Ferri et al. (43) | No—study did not include information on SUDEP | Yes | Unclear—however, the size of the study group did limit the comparisons between right vs. left side EEG abnormalities | Yes | Yes | Yes | Yes | Yes | Yes—possible influence of ASMs on HRV was indicated | N/A | Unclear—not statement was provided |
| Yang et al. (44) | No—no data concerning SUDEP was included in the study | Yes | Unclear -although the authors do acknowledge the small sample size that limits the generalizability of the study findings | Yes | Yes | Yes | Yes—frequency domain HRV analysis | Yes | Yes—differences in hemisphere effects were mentioned | N/A | Unclear—no information was provided |

*ASMs, Anti-Seizure Medications; ECG, Electrocardiogram; EDA, Electrodermal Activity; EEG, Electroencephalograph; GTC, Generalized Tonic Clonic; HRV, Heart Rate Variability; PGES, Post-ictal Generalized Electroencephalographic Suppression; N/A, Not Applicable; SUDEP, Sudden Unexpected Death in Epilepsy.*

**Ratings were: Yes (fully meeting the criterion), No (not meeting the criterion), Unclear (unclear to whether the criterion was met), and N/A (criterion was not applicable) as previously described (20).*

limited, the best way to reduce the risk of SUDEP in children is to optimize the management of epilepsy (33). This principle would be especially relevant in patient groups who are particularly more vulnerable to seizures such as those with RTT (14, 17, 52).

## DISCUSSION

The findings from the systematic review showed that children with epilepsy have (I) an altered sympathovagal tone, (II) have discernible pre- and post-ictal autonomic changes, and (III) have suggestive biomarkers of autonomic dysregulation namely changes in EDA and QTcD. While none of the studies provides direct information relating to pediatric SUDEP, there is some indication that pre- and post-ictal autonomic features could be predisposing risk factors for SUDEP. In line with this view, we wanted to extrapolate the current findings to see if it would provide useful information concerning the management of SUDEP in patients with RTT. We are cognisant of the fact that it would be difficult to predict the onset of epileptiform events in patients with RTT without formal ambulatory or video EEG assessment. This is due to the nature of non-epileptic vacant spells that occur in patients with RTT (52). The characteristic stereotypical movements such as hand movements and dystonia can also make identification of epilepsy in Rett patients more difficult (53). Moreover, another study showed that non-epileptic episodes can also consist of laughing, pupillary dilation and breathing dysregulation (14). This also aligns with the finding that even though seizures are common in RTT, many suspected seizures do not show characteristic epileptiform events on video EEG monitoring (54).

In Rett patients, it is not understood why some seizures are followed by suppression of electrical brain activity on the EEG (PGES). It is possible in RTT, that this could be down to a random event; however, some others have proposed that the events leading to a seizure might also be predictable (55) and be dependent on autonomic mechanisms. In this context, we wanted to address the following questions:

1. What do the autonomic characteristics of SUDEP in children tell us about possible SUDEP in patients with RTT?

2. Can autonomic indices be used to develop biomarkers to identify clinical risk factors of SUDEP in RTT?

### What Do the Autonomic Characteristics of SUDEP in Children Tell Us About Possible SUDEP in Patients With RTT?

At present, information on the autonomic events that could precipitate SUDEP in patients with RTT is unknown. We have previously alluded that patients with RTT could be more vulnerable to changes that could lead to SUDEP (20), however, in RTT it is unclear whether the risk of SUDEP changes across the age range. In RTT, the patterns of seizures come and go, and can be sporadic. If indeed focal epilepsy appears to be more frequent than generalized epilepsy in RTT (52), then it might be possible to detect changes in sympathovagal tone and distinguish focal from generalized seizures as described previously (30).

In the British Isle survey of 137 RTT patients, of the 89 subjects that responded the prevalence of epilepsy was 67 and 62% of patients had GTCs (52). Epilepsy severity data from 736 patients from the Rett Networked Database showed that 55% had seizures classified as grade 1, i.e., well-controlled, and about 32% were judged to be of grade 2 (uncontrolled seizures). GTCs were present in about 46% of patients (17) while in the Natural History Study, ∼46% of patients had focal onset seizures while generalized seizures were noted in 47% of patients (14). These findings indicate that GTC seizures are quite common in patients with RTT, however, there is no empirical evidence to determine what the likelihood of PGES occurring following a generalized convulsive seizure (GCS) in RTT. In the EEG, cessation of background activity is indicative of PGES, and it is during this post-ictal state that patients are at most risk to abnormal cardiorespiratory events (56). A recent evidence synthesis has indicated that in RTT there is a diffuse reduction in the background EEG activity (57) but whether this reduction in background activity meets the threshold of PGES in Rett patients is unknown. The findings from the current review show that (I) in children with epilepsy, PGES is not an uncommon finding, (II) PGES is associated with peri-ictal tachycardia and hypoxemia, (III) pre-ictal LF and HF are higher in children with GCS and PGES, and (IV) post-ictal RMSSD was elevated in children with GCS and PGES compared to those with GCS alone. In children, PGES is also associated with higher scores on the SUDEP-7 inventory.

These findings have implications for patients with RTT because given the underlying brainstem vulnerability and electrical instability of the cardiovascular system, there is a risk of PGES occurring following generalized seizures in this patient group. Whether PGES could increase the risk of SUDEP is a matter of debate (47–49). However, it is probable that patients with RTT could be more vulnerable due to the underlying autonomic cardiorespiratory dysfunction alongside generalized seizures. In the MORTEMUS study, SUDEP cases had a characteristic pattern of respiratory distress, PGES, and then apnoea followed by bradycardia (56). In RTT, abnormal EEG activity can also occur without obvious clinical seizures (52) and this is important because both convulsive and non-convulsive seizures tend to change cardiorespiratory function (58). Even though, post-ictal tachycardia and hyperventilation took longer to return to baseline in convulsive seizures (58), in RTT patients the underlying autonomic dysregulation may further exacerbate the post-ictal tachycardia and hyperventilation, even in those patients with no overt signs of clinical seizures.

Children with GCS and PGES have higher SUDEP-7 inventory scores (29). There is also a correlation between the duration of PGES and age (36). This suggests that adults would be at higher risk of SUDEP; however, it is unknown if this would also be the same for the RTT population. We have surmised that the ANS in children with RTT could be particularly more sensitive to autonomic changes (59). Children with PGES do have an abnormal sympathovagal tone characterized by a greater post-ictal sympathetic activation. During the pre-ictal period of children with GCS and PGES, the time domain parameters of LF and HF are raised (28). These HRV indices reflect changes

in parasympathetic and sympathetic vagal tone, and in the most vulnerable RTT patients, it would be prudent to monitor LF and HF changes to detect early signs of abnormal vagal tone, which could help to manage the risk in these patients.

## Can Autonomic Indices Be Used to Develop Biomarkers to Identify Clinical Risk Factors of SUDEP in RTT?

It is clear from the findings that in children with epilepsy and PGES, there is a modulation in sympathovagal tone (28). The raised LF and HF power before seizure onset suggest increases in sympathetic and parasympathetic tone. Interestingly, the RMSDD, which is a measure of parasympathetic tone, was sustained during the post-ictal period in children with PGES. This sustained increase in RMSDD points toward an increased parasympathetic modulation. Metrics of HRV can also provide information on the seizure phenotype. Children with generalized seizures show a trend of lower RMSSD but higher LF and LF/HF before the seizure (30). In RTT patients, there is some evidence of lower RMS (60). Changes in EDA could also provide useful information on seizures since when compared to adults, children with epilepsy were found to have a higher EDA response (36). We have previously shown that the EDA is disordered in RTT patients (61, 62). In our recent evidence synthesis (59), we proposed that EDA could also be useful in monitoring the physical health of the patient, and we can now extend its use to provide valuable information on the sympathetic response in RTT patients who are more prone to frequent seizures.

While the incidence of SUDEP in patients with RTT is unclear what the current evidence synthesis tells is that given the changes in the sympathovagal tone, Rett individuals could be more susceptible to autonomic changes before and during a seizure. It is clear from the evidence in children with epilepsy that an autonomic derangement occurs leading to fluctuations in sympathetic to parasympathetic shifts and vice versa. It is also apparent that there are characteristic autonomic changes in the pre-ictal period that extend into the post-ictal period. **Figure 3** presents a summary of these changes. In RTT, it should also be borne in mind that the event of SUDEP will also depend on other measures of susceptibility such as the associated post-ictal cardio-respiratory state, and hence the events leading to SUDEP would be very difficult to predict. Despite these limitations, it would be useful to monitor SUDEP-7 ratings, HRV metrics and EDA in RTT patients to see we if can identify patterns in the autonomic dysregulation before and after seizure onset. While we would not be able to determine why some of the seizures in Rett patients progress to SUDEP, this strategy might allow the risk stratification of the most vulnerable patients especially in instances where the presence of clinical seizures is

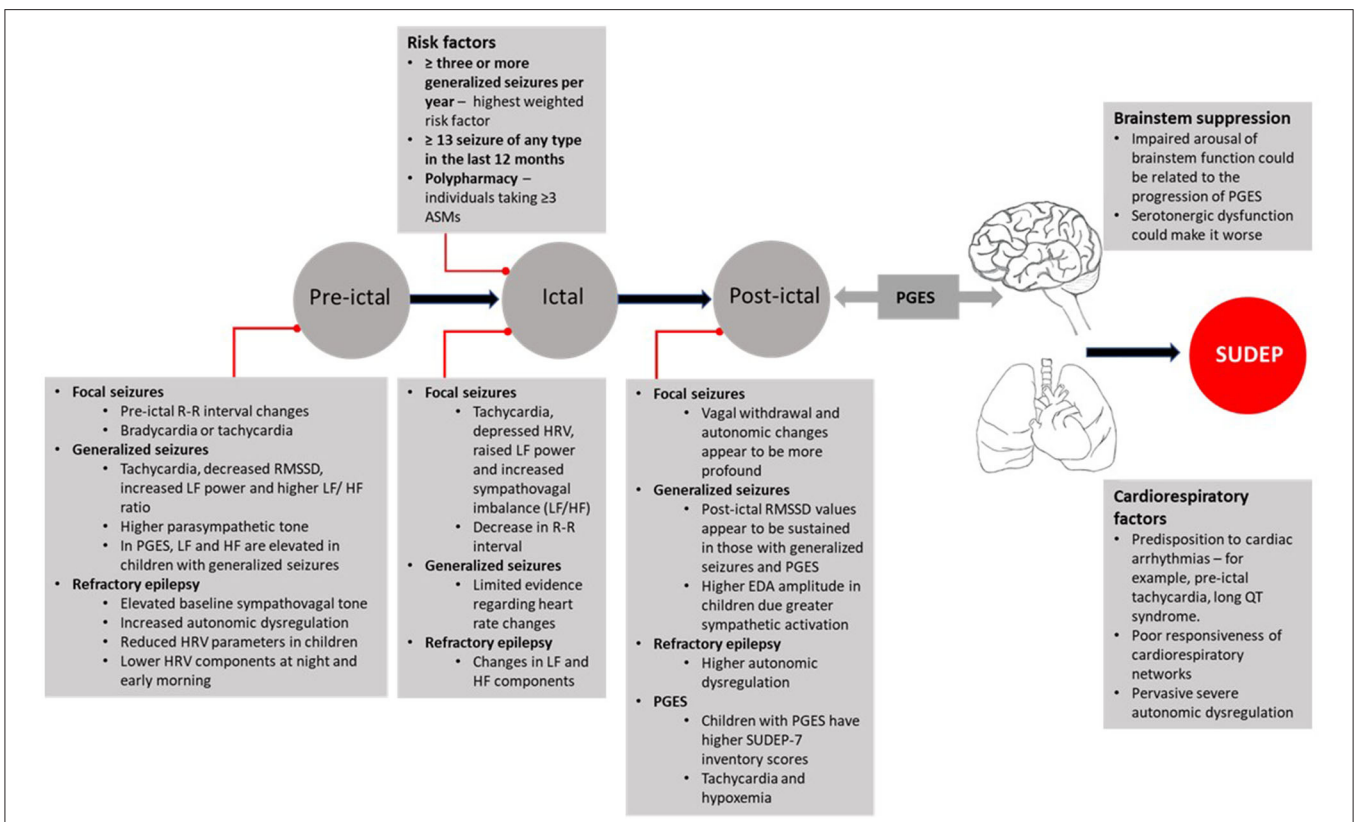


**FIGURE 3 |** Autonomic characteristics of pre-ictal, ictal, post-ictal states, and propensity to SUDEP. ASMs, Anti-Seizure Medications; EDA, Electrodermal Activity; HF, High Frequency; HRV, Heart Rate Variability; LF, Low Frequency; PGES, Post-ictal Generalized Electroencephalographic Suppression; QT, Q and T waves on ECG [electrocardiogram]; RMSSD, Root Mean Square of Successive Differences; R-R, Inter-Beat Interval; SUDEP-7, Sudden Unexpected Death in Epilepsy Risk Inventory.

not obvious and epileptiform activity by EEG monitoring is not readily available.

## CONCLUSION

This is the first study that had conducted a quality appraisal and thematic analysis on the autonomic characteristics of SUDEP in children. While direct evidence regarding studies on pediatric SUDEP is low, the information learned from this systematic review does allow further understanding of the autonomic profile in pediatric epilepsy and the events that could lead to SUDEP. This information is useful for optimizing the management of epilepsy in patients with RTT because it provides evidence on how important it is to obtain the best seizure control in this patient group.

Pediatric SUDEP is heterogeneous and likely to be driven by a range of factors. Brainstem suppression could be a common mechanism (4), however, it is less certain how events in the early pre-ictal phase develop into a more severe post-ictal phase and in some instances to death. Serotonergic dysfunction could play a role. Recently in a prospective multicenter study of SUDEP in 49 patients, it was shown that higher levels of post-ictal serum 5-HT were associated with reduced seizure related breathing dysregulation and this increase might protect against the deleterious changes leading to SUDEP (63).

The present evidence synthesis suggests that in children with epilepsy, the sympathovagal balance is impaired and there are also subtle changes in autonomic characteristics pre- and post-seizure onset. In patients with RTT, the epilepsy is likely to cause fluctuations in HRV and EDA because of the dysregulation in the central autonomic network. Following the onset of a seizure, in typical circumstances, there would be a decrease in vagal tone with a concomitant increase in heart rate (39), however the underlying autonomic dysregulation in RTT would lead to fluctuations in this vagal tone, and this could predispose patients to sympathetic storming, which would have an impact on the ascending control of brainstem functions. In Rett patients there are serotonergic abnormalities (20) and serotonergic agents have been shown to reduce SUDEP in animal models (64). As post-ictal cardiorespiratory states are less agile in RTT, one possible option would be to consider serotonergic agents especially in Rett patients whose seizures are poorly controlled to see if the recovery time post-seizure can be reduced. Robust clinical trials would be needed to test this hypothesis specially in the RTT patient population.

Previous data suggest that GTC seizures are the most common seizure phenotype in SUDEP (21, 22, 65). In RTT the frequency of generalized seizures range from 62 to 46% (14, 17, 52); however further work would be needed to identify the most common seizure phenotype that leads to SUDEP in RTT and also why some seizures in RTT terminate while some might eventually lead to SUDEP. Desynchronization in seizure mechanisms could result in a summation of events that cause brainstem shutdown in SUDEP (66) and the current findings show that after seizure onset, RMSDD remains sustained in children with generalized convulsive seizures and PGES (28) suggesting a more severe autonomic dysregulation post-seizure. Increases in EDA have also been documented during the post-ictal period (36, 67) and this increase could provide critical information alongside HRV measurements in Rett patients. Measurement of EDA using a non-invasive wearable sensor offers an alternative way to monitor seizure outcomes in RTT patients and could optimize the management of seizures in this vulnerable patient group. This would also help to support EEG findings for the diagnosis of epilepsy, as in RTT, the EEGs can be abnormal even when there are no seizures (14). Typically, EEGs would need to be performed during an 'event', but because these EEGs are usually performed in a clinical setting, infrequent seizures could be missed. In this scenario, the use of wearable sensors to detect subtle pre-ictal changes in EDA and HRV (alongside EEG monitoring) would be beneficial in this patient group.

In summary, the limitation in identifying direct inferences regarding SUDEP underscores the need for further research on this topic in the pediatric population. Even though the mechanism leading to SUDEP are likely to be complex and involve post-ictal cardiorespiratory mechanisms, in vulnerable populations where there is already an autonomic dysregulation, monitoring the autonomic characteristics of EDA and HRV pre- and post-seizure onset using non-invasive wearable sensors would be beneficial for managing the SUDEP risk.

## LIMITATIONS

The level of evidence regarding the autonomic characteristics of pediatric SUDEP is low, as there has been no specific study of SUDEP in RTT, and the extrapolation of information to the broader RTT community should be placed in this context. While the review shows that autonomic characteristics would be useful in managing epilepsy risk in RTT, given the probabilistic nature of SUDEP, the findings from the present study should be interpreted with caution because they do not show that monitoring these autonomic characteristics would prevent SUDEP. We have also proposed that autonomic dysregulation follows a non-linear trajectory (20) and given that the time course of epilepsy in RTT fluctuates across the lifespan (14, 17, 52), HRV and EDA measurements would not be uniform, and without higher-order analytics it would be difficult to predict autonomic features of seizure onset across different patient sub-groups. The patients in the studies evaluated were also on different ASMs, and we cannot exclude the role ASMs would have had on the trajectory of autonomic dysregulation and whether these medications influence HRV.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

JS developed the idea of the study and wrote it. The systematic searches of the databases were undertaken by JS

and EL in an independent and blinded manner. EL reviewed the thematic analysis and quality appraisal of the articles. Both EL and PS reviewed the scientific content of the draft and final versions. All authors have read and approved the final manuscript.

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**Conflict of Interest:** PS was a Principal Investigator (PI) on the Sarizotan (Protocol Number Sarizotan/001/II/2015; ClinicalTrials.gov Identifier: NCT02790034) and is currently the PI for the Anavex Life Sciences Corp. (Protocol Number: ANAVEX2-73-RS-002) clinical trial in Rett Syndrome (RTT). PS is the co-inventor of the HealthTracker$^{TM}$ and is the Chief Executive Officer and shareholder in HealthTracker$^{TM}$. JS was a Trial Research Methodologist on the Sarizotan Clinical Trial (Protocol Number Sarizotan/001/II/2015; ClinicalTrials.gov Identifier: NCT02790034) in patients with RTT and is a Research Manager on the Anavex Life Sciences Corp. (Protocol Number: ANAVEX2-73-RS-002) clinical trial for RTT. JS is also an advisor for Reverse Rett.

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# Nicotinic Receptors in the Brainstem Ascending Arousal System in SIDS With Analysis of Pre-natal Exposures to Maternal Smoking and Alcohol in High-Risk Populations of the Safe Passage Study

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Pre-natal exposures to nicotine and alcohol are known risk factors for sudden infant death syndrome (SIDS), the leading cause of post-neonatal infant mortality. Here, we present data on nicotinic receptor binding, as determined by $^{125}$I-epibatidine receptor autoradiography, in the brainstems of infants dying of SIDS and of other known causes of death collected from the Safe Passage Study, a prospective, multicenter study with clinical sites in Cape Town, South Africa and 5 United States sites, including 2 American Indian Reservations. We examined 15 pons and medulla regions related to cardiovascular control and arousal in infants dying of SIDS ($n = 12$) and infants dying from known causes ($n = 20$, 10 pre-discharge from time of birth, 10 post-discharge). Overall, there was a developmental decrease in $^{125}$I-epibatidine binding with increasing postconceptional age in 5 medullary sites [raphe obscurus, gigantocellularis, paragigantocellularis, centralis, and dorsal accessory olive ($p = 0.0002$–$0.03$)], three of which are nuclei containing serotonin cells. Comparing SIDS with post-discharge known cause of death

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(post-KCOD) controls, we found significant decreased binding in SIDS in the nucleus pontis oralis ($p = 0.02$), a critical component of the cholinergic ascending arousal system of the rostral pons (post-KCOD, 12.1 ± 0.9 fmol/mg and SIDS, 9.1 ± 0.78 fmol/mg). In addition, we found an effect of maternal smoking in SIDS ($n = 11$) combined with post-KCOD controls ($n = 8$) on the raphe obscurus ($p = 0.01$), gigantocellularis ($p = 0.02$), and the paragigantocellularis ($p = 0.002$), three medullary sites found in this study to have decreased binding with age and found in previous studies to have abnormal indices of serotonin neurotransmission in SIDS infants. At these sites, $^{125}$I-epibatidine binding increased with increasing cigarettes per week. We found no effect of maternal drinking on $^{125}$I-epibatidine binding at any site measured. Taken together, these data support changes in nicotinic receptor binding related to development, cause of death, and exposure to maternal cigarette smoking. These data present new evidence in a prospective study supporting the roles of developmental factors, as well as adverse exposure on nicotinic receptors, in serotonergic nuclei of the rostral medulla—a finding that highlights the interwoven and complex relationship between acetylcholine (via nicotinic receptors) and serotonergic neurotransmission in the medulla.

**Keywords: acetylcholine, serotonin, cardiorespiratory, arousal, medulla oblongata, mesopontine tegmentum**

## INTRODUCTION

The sudden infant death syndrome (SIDS) is a major worldwide public health problem. It is defined as the sudden death of a seemingly healthy infant under 1 year of age that remains unexplained after a thorough case investigation, including the performance of a complete autopsy, an examination of the death scene, and a review of the infant's clinical history (1). Death typically occurs during sleep or during one of the many transitions to arousal that occur in normal infant sleep (2). SIDS is the leading cause of post-neonatal infant death in the United States where the overall rate is 0.35/1,000 live births (3). The SIDS risk increases in socioeconomically disadvantaged minority populations throughout the world, e.g., African-Americans in the urban United States, American Indians in the Northern Plains, mixed ancestry groups in Cape Town in South Africa, Maoris in New Zealand, and Aboriginal and Torres Strait Islanders in Australia (3–7). Biological mechanisms in minority high-risk SIDS populations have been historically understudied because of the decreased access to modern forensic centers with pediatric research tools, lack of funds for research in health disparities, and the general mistrust of autopsy by these minority populations (8–10).

A leading hypothesis in SIDS research today is that there is an abnormality in neurotransmitter networks in the lower brainstem that regulate cardiorespiratory control and arousal (11). We and others have reported abnormalities in tissue parameters of the neurotransmitter serotonin (5-HT) in the serotonergic homeostatic network in the medulla oblongata (lower brainstem) in SIDS cases compared to controls (12–17) as well as abnormalities in cholinergic (18–25), GABAergic (26), and substance P (27) networks. These abnormalities likely impair protective reflexes to life-threatening challenges during a sleep period, leading to defective arousal to a metabolic stressor (hypoxia, hypercarbia) and sleep-related sudden death. The underlying premise is that a vulnerable infant with a biological defect in homoeostasis dies suddenly in a sleep period when they fail to respond to an exogenous stressor in a critical developmental period (the Triple Risk model) (28). While the origin or basis of the biological defect is unknown, one possibility includes altered development of neurotransmitter systems due to exposure to adverse conditions *in utero*. Among these exposures, pre-natal exposure to nicotine and alcohol are candidates based on epidemiological data showing the contribution of maternal smoking (29–31) and drinking (32) to SIDS risk. Most recently, the Safe Passage Study conducted by the Pre-natal Alcohol in SIDS and Stillbirth (PASS) Network (see below) reported an increased relative risk for SIDS of 4.86 (95% CI: 0.97–24.27) for infants with pre-natal exposure to smoking only beyond the first trimester, as compared to those unexposed or those whose mothers reported quitting early in pregnancy (33). The relative risk increased to ∼12-fold (98% CI: 2.59–53.7) in infants whose mothers reported both smoking and drinking beyond the first trimester, suggesting a combined, possibly synergistic, effect on infant risk (33).

The Safe Passage Study was a large, prospective, multidisciplinary study designed, in part, to investigate the association between pre-natal alcohol and/or pre-natal smoke exposure, and SIDS and stillbirth (34). A key objective of the Safe Passage Study was to elucidate the role of pre-natal exposures in altered development of neurotransmitter systems in the human brainstem. This includes development of the cholinergic receptor system in cardiorespiratory and arousal brainstem sites involved in homeostatic regulation. Acetylcholine is a neurotransmitter that mediates its effects via 2 classes of cholinergic receptors, metabotropic muscarinic

receptors, and ionotropic nicotinic acetylcholine receptors (nAChRs), localized diffusely throughout the brain and brainstem. Early in fetal development, acetylcholine, via interactions with acetylcholine receptors, serves as a growth factor, affecting neuronal proliferation, growth, survival, differentiation, and pathfinding (35). Aberrant embryonic alterations in acetylcholine signaling adversely affects its morphogenetic properties during development with lasting effects into the post-natal period (35). In the Safe Passage Study, we have focused specifically on acetylcholine signaling as mediated by nAChRs because of the agonistic properties of nicotine upon binding to this receptor subtype and because of the documented effect of nicotine on nAChRs expression (36).

Nicotinic receptors are ligand-gated cation channels that exist as pentamers of subunits around a central pore. Genes encoding a total of 16 subunits (α1–10, β1–4, δ, ε, γ) have been identified in mammals (37). They are present as either homopentamers (α7, α9) or heteropentamers throughout the central and peripheral nervous system and can be found both at pre- and post-synaptic membranes (38). Human developmental studies have shown expression of many subunits as early as 4 to 5 gestational weeks, with high expression during early to midgestation, including within the brainstem (39). During early infancy, nAChR expression decreases substantially (40), suggesting a heightened vulnerability to the effects of maternal smoking during gestation. Maternal smoking results in nicotine crossing the placental barrier, the fetal blood–brain barrier, and binding to the endogenous nAChRs in the fetal brain (41) affecting nAChR expression and function. Similarly, ethanol from maternal drinking crosses the placenta into the fetal circulation and interacts with nAChRs via an ethanol binding pocket to modulate the action of the receptor (42–46). Given that both nicotine and ethanol affect the action of nAChRs within the brain, these receptors represent a common target underlying the adverse effect of maternal smoking and drinking during pregnancy. Previous studies have examined the effects of pre-natal exposures on nAChRs in the brain or brainstem of autopsied infants and in relationship to SIDS (19, 22–25), including in the American Indian population, which is at a high risk for pre-natal exposure and SIDS death (21). These studies were, however, retrospective in nature with exposure information collected at autopsy. The strengths of the Safe Passage Study include its prospective design and rigorous assessment of quantity, frequency, and timing of pre-natal alcohol and smoking exposures. It is also uniquely focused on brainstem analysis in at-risk SIDS minority populations. Using cases collected from the Safe Passage Study and receptor ligand autoradiography, we tested the 2-fold hypothesis that (1) nAChR binding, as determined by binding to nAChR agonist $^{125}$I-epibatidine, is significantly altered in medullary centers and pontine sites related to cardiorespiratory function and arousal in SIDS infants compared to controls and (2) that pre-natal exposure to alcohol and smoking modifies $^{125}$I-epibatidine binding in these same brainstem sites.

# MATERIALS AND METHODS

## Design of the Safe Passage Study

The study's hypotheses, specific aims, common protocol, enrollment, shipping, compliance, and specimen donation have been described in detail (34), as well as the approach to autopsy consent in socioeconomically disadvantaged populations (47). In brief, the Safe Passage Study was an international prospective, multicenter longitudinal cohort study with data collection conducted between August 2007 and October 2016. Clinical sites were selected based upon known high rates of maternal drinking and smoking during pregnancy and known high rates of SIDS in the population; however, all women from the catchment areas presenting for care at these sites were eligible to participate. These predominately include pregnant (1) American Indian and Caucasian women from the Northern Plains and (2) mixed ancestry women of the Western Cape, South Africa. Screening and enrollment occurred at pre-natal clinics affiliated with each clinical site between 6 weeks gestation up to, but not including, delivery. The maternal and fetal/infant dyads were followed during pregnancy and from birth until infants were 1 year of age, i.e., the risk period of SIDS. Detailed information regarding quantity, frequency, and timing of substance use was self-reported up to 4 times during pregnancy (recruitment, 20–24, 28–32, and 34+ gestational weeks) and at 1 month post-delivery. At sites in South Africa, a medico-legal autopsy was performed upon demise. Consent for research was sought from the family as soon as possible after death (47). At sites within the United States, an autopsy was regularly ordered by the coroner/medical examiner after which the family was approached for consent to the donation of tissue for research purposes. If consent was given, brain portions were frozen and shipped on dry ice to the Developmental Brain and Pathology Center (DBPC), Department of Pathology, Boston Children's Hospital, the centralized laboratory for research analysis (48). The study, including the use of brain tissue, was approved by the Institutional Review Boards (IRBs) of the local hospitals at which the infants were autopsied and Boston Children's Hospital. When designed, the Safe Passage Study for brain analysis anticipated and was powered on 37 SIDS cases and 37 non-SIDS controls, which would allow detection of a difference in receptor binding levels as small as 0.67 standard deviation.

## Clinical Database

SIDS was defined using a study definition that included the sudden unexpected death of an infant, <1 year of age, whose cause of death remained unexplained after review of all available information, including performance of a complete autopsy, examination or report of the death scene, and review of the clinical history (1). In addition, SIDS included deaths that might otherwise have been classified as undetermined including infants dying in unsafe sleep conditions but without evidence of mechanical asphyxia or suffocation by overlay. Known cause of death (KCOD) controls were defined as infants whose cause of death was identified after review of all available information (33). All demises were adjudicated by a team of

pediatric pathologists, neuropathologists, forensic pathologists, a neonatologist, geneticist, obstetrician, and developmental psychologist who were blinded to pre-natal exposures. Case reviews did include toxicology, genetic testing (when appropriate and available), and metabolic testing (when appropriate and available). The postmortem interval (PMI) was calculated as the time when the infant was last seen alive to proclamation of time of discovery, as in previous studies by us (16, 49). Prospective collection of demise cases in the Safe Passage Study included 28 SIDS and 38 control cases that died after discharge from the hospital (post-discharge) (33). Of these cases, 12 SIDS and 10 post-discharge KCOD (post-KCOD) controls were available for neurochemical studies. There were 16 SIDS and 28 post-KCOD cases that were accrued in the Safe Passage Study but not available for neurochemistry either due to a lack of consent for autopsy research or due to technical issues related to quality of tissue. There were 45 KCOD controls that died prior to leaving the hospital after delivery [pre-discharge KCOD (pre-KCOD)] and 10 of these were analyzed for $^{125}$I-epibatidine binding to provide baseline developmental data.

## Brainstem Accrual

At autopsy, the brain was removed, weighed fresh, and examined for gross developmental and acquired abnormalities. The entire brainstem was removed from the level of the midbrain at the mammillary bodies to the cervicomedullary junction in 1.5-cm samples, sectioned on a Leica motorized cryostat at 20 μm, mounted on glass microscopic slides, and stored at −80°C until used for tissue receptor autoradiography. Frozen blocks were stored at −80°C in air-tight plastic containers.

## $^{125}$I-Epibatidine Binding and Generation of Brainstem Autoradiograms

To examine nicotinic receptors, we used receptor ligand autoradiography. Unlike homogenate radioligand binding, autoradiography allows the visualization of spatial anatomy and provides details of regional expression patterns (50). It is quantitative in nature and thus provides benefits over other techniques like immunohistochemistry. Radioligand $^{125}$I-epibatidine was used for autoradiographic analysis. Epibatidine is a nAChR agonist with high affinity to α4β2 nAChRs, one of the major subtypes of nAChRs in the brain, and with lower affinity to α7 nAChRs (51). The autoradiography procedures were performed according to the detailed methodology previously reported from our laboratory for $^{3}$H-epibatidine (52), with modification for the iodinated ligand. All steps were performed at room temperature. In brief, total $^{125}$I-epibatidine binding was determined by incubation of the frozen, unfixed sections with 0.5 nM $^{125}$I-epibatidine (2200 Ci/mmol, PerkinElmer, Waltham, MA, USA) in binding buffer consisting of 50 nM Tris–HCl, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4 for 60 min, an incubation time sufficient for equilibrium to be reached in epibatidine binding experiments (53). Non-specific binding was determined in adjacent sections by addition of 0.5 nM $^{125}$I-epibatidine and 300 μm of L-nicotine bitartrate. To remove unbound ligand, the sections were washed in a series of buffer changes (5 min each) followed by 3 dips in distilled water. Sections were then left overnight for drying, after which they were placed in cassettes and exposed to a BAS_TR2025 phosphoimaging plate (GE Healthcare Life Sciences, Marlborough, MA) for 20 h, with a set of $^{125}$I standards (American Radiolabeled Chemicals, Inc, MO, USA) calibrated by the manufacture in terms of radioactivity per unit weight. The standards allowed for the conversion of relative optical density to fentamoles per milligram (fmol/mg) of tissue to determine binding levels. A BAS-500 Bioimaging Analyzer (Fuji-Film) with Image Reader version 1.8 software (FujiFilm) was used to generate digital autoradiographic images from phosphoimaging plates. Quantitative densitometry of autoradiograms was performed using a MCID 5+ imaging system (Imaging Research). Specific receptor binding was determined by subtracting non-specific binding from total binding in individual tissue sections. The same sections incubated for autoradiography were subsequently stained with hematoxylin and eosin for anatomical assessment.

## Analysis of $^{125}$I-Epibaditine Binding in Homeostatic Brainstem Sites

For relevance, we provide detail of the neuroanatomical functions and connectivity of the medullary and pontine nuclei studied here (see **Supplemental Material**). The human brainstem sites measured (**Figure 1**) were defined with reference to Olszewski and Baxter brainstem atlas (54) and confirmed with Paxinos and Huang brainstem atlas (55). For each case, $^{125}$I-epibatdine binding in the brainstem was measured at 3 levels; mid medulla at the level of nucleus of Roller, which included the nucleus of the solitary tract (NTS) (all visceral sensory inputs of the autonomic nervous system and sympathetic autonomic system integration), the hypoglossal nucleus (HG) (airway patency, especially during sleep), dorsal motor nucleus of the vagus (DMX) (preganglionic vagal outflow of the parasympathetic autonomic nervous system), centralis (CEN), principal inferior olive (PIO), and medial accessory olive (MAO) (the cerebellar network); rostral medulla at the level of the nucleus pre-positus, which included the raphe obscurus (RO), gigantocellularis (GC), paragigantocellularis lateralis (PGCL), core nuclei of serotonergic homeostatic medullary network, and the dorsal accessory olive (DAO); and rostral pons at the level of nucleus parabrachialis lateralis, which included the locus coeruleus (LC) (major source neurons of the noradrenergic ascending arousal network), nucleus pontis oralis (PoO) (part of source neurons of the cholinergic ascending arousal system), griseum pontis (GRPo) (pre-cerebellar nucleus, part of the pontocerebellar network), and median raphe (MR) (part of the rostral 5-HT ascending arousal network) (**Figure 1**). With the exception of the midline nuclei (RO and MR), $^{125}$I-epibatidine binding was measured from both sides (left and right) of the section and the means calculated to determine final value in fmol/mg.

## Collection of Exposure Data

This study used a modified timeline follow-back method to collect exposure related to maternal smoking/drinking during pregnancy (56). At each visit during and after pregnancy, the participant was asked about the last date of use (separately for

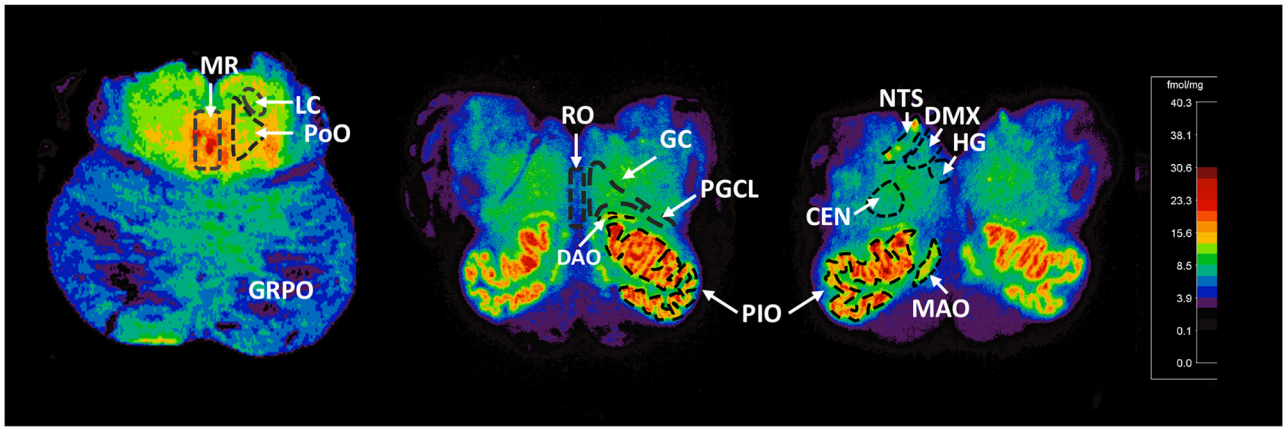


**FIGURE 1 |** Representative distribution of $^{125}$I-epibatidine binding in the pons and medulla. Illustrative autoradiograms displaying $^{125}$I-epibatidine binding in tissue sections at the level of the pons (left), rostral medulla (middle), and mid medulla (right). Sections are taken from a 52-postconceptional week SIDS case (pons) and a 45-postconceptional week SIDS case (rostral and mid medulla) case. Receptor binding is normalized to the same scale (shown). The amount of receptor binding in fmol/mg is indicated with color according to the scale. The nuclei measured are denoted with dashed boundary lines and labeled. These nuclei include the following: (Pons) MR, median raphe; LC, locus coeruleus; PoO, nucleus pontis oralis; GRPO, griseum pontis; (Rostral Medulla) RO, raphe obscurus; GC, gigantocellularis; PGCL, paragigantocellularis lateralis; PIO, principal inferior olive; DAO, dorsal accessory olive; (mid medulla) HG, hypoglossal nucleus; DMX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; CEN, centralis; MAO, medial accessory olive.

alcohol and smoking). For data relating to alcohol, they were asked about consumption for ±15 days around last menstrual period, as well as 30 days prior to the last drinking day since their last research appointment. For smoking, they were asked about the frequency of smoking and number of cigarettes on a typical day for the 30 days prior to the last date of use since their last research appointment. To estimate the total number of drinks consumed during pregnancy, each drink consumed was first standardized where 1 drink is defined as 14 g of ethanol. The study design did not allow consumption data to be collected on every single day of pregnancy, so missing values were imputed using the k-nearest neighbor (kNN) method. Methods for alcohol imputation are cited elsewhere (57). Since frequency of cigarette use was collected more sparsely, average cigarettes smoked per week during pregnancy was used. The number of cigarettes smoked each week during pregnancy was calculated, and missing weeks was imputed in a similar way to the alcohol imputation. After imputation, an average number of cigarettes during pregnancy was calculated.

## Statistical Analysis

Descriptive analysis was conducted to analyze differences between cause of death (SIDS vs. KCOD controls) and demographics, maternal substance use during pregnancy, infant sleep practices, and relevant autopsy and clinical findings. Analyses used included Student's *t*-test or Mann–Whitney *U* for continuous variables and chi-square testing with Fisher's exact test for categorical variables. Maternal demographics assessed included age, education, housing type, history of loss by SIDS, and delivery type. Infant demographics assessed included birth weight and length, gestational age at birth, post-natal age at death, gender, and race. Autopsy findings assessed included postmortem interval, body weight at autopsy, and brain weight at autopsy. Maternal use of alcohol and smoking during pregnancy were assessed as binary values (used during pregnancy or not) and as continuous values (total number of drinks during pregnancy and average number of cigarettes per week). Maternal use of alcohol and smoking by trimester was assessed as continuous values (number of drinks by trimester and average cigarettes per week). Infant sleep practices assessed included sleep position last placed, sleep position found, and whether or not the infant was covered by bedding or blankets.

Multivariate linear regression models were built to analyze differences in mean $^{125}$I-epibatidine binding values by case diagnosis and exposure. Post-conceptional age (PCA) was controlled for in all models, as it is significantly different by case diagnosis and associated with $^{125}$I-epibatidine binding. A subanalysis assessed the effect of development (PCA) on $^{125}$I-epibatidine binding in the 10 pre-KCOD- and 10 post-KCOD-control infants. There was no effect of PMI on binding; therefore, PMI was not controlled for in the analyses. $p < 0.05$ were considered statistically significant. Analyses were conducted using SAS 9.4.

# RESULTS

## Clinicopathological Information

The demise cohort for $^{125}$I-epibatidine analyses include SIDS ($n = 12$), post-KCOD controls ($n = 10$), and pre-KCOD controls ($n = 10$) (see above). The causes of death for the control groups are given in **Table 1**, with demises separated as pre- or post-discharge, based on whether the infant died in the hospital without being discharged after birth or at home after discharge. Selected demographic data including incidence of exposure are summarized in **Table 2**. Pre-KCOD cases were included only for the purpose of looking at developmental

**TABLE 1 |** Causes of death in pre- and post-discharge known cause of death (KCOD) cases.

| Case | Pre- or Post-discharge | GA (wks) | PNA (wks) | PCA (wks) | Cause of death |
|---|---|---|---|---|---|
| 1 | Pre-discharge | 25.7 | 0.1 | 25.8 | Complications of pre-maturity |
| 2 | Pre-discharge | 27.0 | 0.04 | 27.0 | Hyaline membrane disease, chorioamnionitis and placental abruption |
| 3 | Pre-discharge | 27.0 | 0.6 | 27.6 | Pre-eclampsia and pre-maturity |
| 4 | Pre-discharge | 30.3 | 1.4 | 31.7 | Omphalocele, peritonitis, sepsis |
| 5 | Pre-discharge | 32.6 | 0.1 | 32.7 | Pulmonary hemorrhage |
| 6 | Pre-discharge | 32.9 | 0.1 | 33.0 | Fetal head trauma due to motor vehicle accident |
| 7 | Pre-discharge | 31.7 | 1.6 | 33.3 | Klebsiella pneumonia, necrotizing enterocolitis, jaundice |
| 8 | Pre-discharge | 37.0 | 0.04 | 37.0 | Intrauterine growth restriction |
| 9 | Pre-discharge | 37.5 | 0.02 | 37.5 | Pulmonary hypoplasia, multicystic dysplastic kidney disease complicating Potter's sequence |
| 10 | Pre-discharge | 41.3 | 0.6 | 41.9 | Meconium aspiration, severe bronchopneumonia, perinatal asphyxia |
| 11 | Post-discharge | 27.3 | 9.4 | 36.7 | Respiratory infection |
| 12 | Post-discharge | 35.6 | 2.1 | 38.0 | Respiratory infection |
| 13 | Post-discharge | 27.6 | 12.9 | 40.5 | CNS infection |
| 14 | Post-discharge | 38.9 | 1.6 | 40.5 | Congenital defects |
| 15 | Post-discharge | 32.1 | 12.1 | 44.2 | Renal; tubule-interstitial nephritis |
| 16 | Post-discharge | 36.0 | 9.0 | 45.0 | Respiratory infection |
| 17 | Post-discharge | 40.0 | 11.7 | 51.7 | Respiratory infection |
| 18 | Post-discharge | 38.3 | 14.1 | 52.4 | CNS infection |
| 19 | Post-discharge | 39.6 | 22.7 | 62.3 | Gastrointestinal infection |
| 20 | Post-discharge | 38.6 | 25.9 | 64.4 | Respiratory infection |

*Pre-discharge cases are infants who died in the hospital prior to being released after birth. Post-discharge cases are infants that died after discharge from the hospital. KCOD, known cause of death; CNS, central nervous system; N, number; GA, gestational age; PNA, post-natal age; PCA, postconceptional age; wks, weeks.*

changes in receptor binding. Pre-KCOD cases ($n = 10$) ranged from 25.8 to 41.9 postconceptional weeks (mean = 32.8 weeks) (**Table 2**). Sixty percent of pre-KCOD cases were male ($n = 6$) and 80% ($n = 8$) were South African mixed race with the other 20% being American Indian ($n = 1$) or Caucasian ($n = 1$). The SIDS cohort was statistically analyzed relative to the post-KCOD cases only. In the comparison between SIDS and post-KCOD, there was no significant difference in mean gestational age (GA), PCA, PMI, birth weight, sex, pre-mature birth, autopsy body, or brain weight (**Table 2**). The majority of cases was from the South Africa clinical site (**Table 2**), accounting for the predominately South African mixed-race assignment in SIDS (92%) and post-KCOD (80%) [$p$ = non-significant (ns)]. We assessed several maternal characteristics and found a significant difference only in education status ($p = 0.03$) with the majority of post-KCOD mothers completing high school (60%) and the majority of SIDS mothers reporting some high school education (67%) (**Table 2**). Other demographic measures were not significantly different between SIDS and post-KCOD controls. These include crowding index (>1 person/room), employed (yes/no), marital status, and housing type (council housing, informal shack/squatter, apartment/house, other) (data not shown).

The presence of infection in KCOD controls, including peripheral and central infection, is noted in **Table 1**. Evidence of mild infection was noted at autopsy in 7 out of 12 SIDS cases including group B *Streptococcus* in the lungs ($n = 1$), inflammatory changes in the larynx ($n = 1$), inflammatory changes in the pharynx ($n = 1$), inflammatory cells in the lamina propria ($n = 1$), mild pneumonitis ($n = 2$), and positive *Clostridioides difficile* in the stool ($n = 1$).

Information regarding maternal smoking and drinking during pregnancy was available for all cases in the SIDS ($n = 12$) and post-KCOD cases ($n = 10$). The incidence of maternal smoking during pregnancy (yes or no) was 100% (12/12) in the SIDS group (**Table 2**) and ranged from an average of 0.1 cigarettes per week to 62.3 (median of 20.6) (**Table 3A**). This was not statistically different from post-KCOD cases whose incidence of smoking was 90% (**Table 2**) and ranged from an average of 0 cigarettes per week to 58.6 (median of 28.7) (**Table 3A**). Maternal smoking was neither statistically different between SIDS and controls in any one trimester nor was it statistically different across trimesters in SIDS or in controls. The incidence of maternal drinking during pregnancy (yes or no) was 50% (6/12) in the SIDS group (**Table 2**) and ranged from 0 drinks during pregnancy to 210.75 drinks in pregnancy (median of 2.6) (**Table 3B**). This was not statistically

**TABLE 2 |** Selected demographic information.

| | Pre-discharge Pre-KCOD | | Post-discharge | | | | |
|---|---|---|---|---|---|---|---|
| | | | SIDS | | Post-KCOD | | *p*-value |
| | *N* | Mean ± STD or *n* (%) or median | *N* | Mean ± STD or *n* (%) or median | *N* | Mean ± STD or *n* (%) or median | |
| Maternal age (yrs) | 10 | 25.8 ± 7.8 | 12 | 27.1 ± 4.9 | 10 | 27.0 ± 6.1 | 0.97 |
| Maternal history of loss by SIDS | 6 | 0 (0) | 11 | 1 (9) | 7 | 0 (0) | 1.00 |
| Caesarian section | 10 | 5 (50) | 12 | 3 (25) | 10 | 3 (30) | 1.00 |
| Maternal education | 9 | | 12 | | 10 | | **0.03** |
| Any primary school | | 0 (0) | | 2 (17) | | 0 (0) | |
| Some HS | | 7 (78) | | 8 (67) | | 4 (40) | |
| Completed HS | | 2 (22) | | 1 (8) | | 6 (60) | |
| Beyond HS | | 0 (0) | | 1 (8) | | 0 (0) | |
| GA (wks) | 10 | 32.3 ± 5.1 | 12 | 36.1 ± 3.4 | 10 | 35.4 ± 4.8 | 0.70 |
| PCA (wks) | 10 | 32.8 ± 5.1 | 12 | 49.0 ± 12.1 | 10 | 47.6 ± 9.8 | 0.76 |
| PMI (h) | 10 | 44.1 ± 34.4 | 12 | 41.6 ± 25.3 | 10 | 47.6 ± 37.1 | 0.66 |
| Birth weight (g) | 10 | 1,590.8 ± 803.4 | 12 | 2,388.3 ± 785.0 | 10 | 2,535.0 ± 1,061.0 | 0.71 |
| Birth length (cm) | 2 | 33.5 ± 3.5 | 10 | 47.4 ± 4.3 | 8 | 47.8 ± 5.7 | 0.88 |
| Male sex | 10 | 6 (60) | 12 | 6 (50) | 10 | 6 (60) | 0.69 |
| Pre-term birth (<37 GA wks) | 10 | 7 (70) | 12 | 7 (58) | 10 | 5 (50) | 1.0 |
| Race | 10 | | 12 | | 10 | | 0.71 |
| American Indian | | 1 (10) | | 0 (0) | | 1 (10) | |
| South African mixed race | | 8 (80) | | 11 (92) | | 8 (80) | |
| Caucasian | | 1 (10) | | 1 (8) | | 1 (10) | |
| Autopsy body wt (g) | 10 | 2,283.3 ± 1,723.7 | 12 | 4„168.7 ± 1,935.7 | 9 | 3672.4 ± 1,870.0 | 0.56 |
| Autopsy brain wt (g) | 9 | 236.2 ± 126.9 | 10 | 510.3 ± 173.9 | 6 | 507.8 ± 238.1 | 0.98 |
| Occipital frontal çircum (cm) | 10 | 29.0 ± 4.9 | 12 | 37.4 ± 4.6 | 9 | 37.5 ± 4.9 | 0.97 |
| Alcohol during pregnancy (y/n) | 8 | 5 (63) | 12 | 6 (50) | 10 | 7 (70) | 0.41 |
| *N* drinks in pregnancy | 10 | 0.9 | 12 | 2.6 | 10 | 8.3 | 0.48 |
| Smoking during pregnancy (y/n) | 8 | 8 (100) | 12 | 12 (100) | 10 | 9 (90) | 0.45 |
| Avg cigarettes/week | 6 | 9.3 | 11 | 20.6 | 8 | 28.7 | 0.90 |

*N represents the number of cases with available demographic information. KCOD, known cause of death; SIDS, sudden infant death syndrome; yrs, years; HS, high school; GA, gestational age; PCA, postconceptional age; PMI, postmortem interval; wks, weeks; hrs, hours; circum; circumference; cm, centimeter; y/n, yes/no; Avg, average; g, grams, STD, standard deviation. Significant p < 0.05 are in bold.*

different from post-KCOD cases whose incidence of drinking was 70% (**Table 2**) and ranged from 0 drinks during pregnancy to 102.5 drinks in pregnancy (median of 8.3) (**Table 3**). There was no statistical difference between SIDS and post-KCOD cases when exposure was analyzed by trimesters (**Tables 3A,B**).

Information regarding sleep-related risk factors was available for all cases within the SIDS group but only 2 of the 10 post-KCOD controls (data not shown). Within the SIDS group, the prevalence of infants last placed supine was 8% (1/12), on their side (lateral) was 33% (4/12), and prone was 58% (7/12). The prevalence of SIDS infants in the demised state on their side (lateral) was 64% (7/11) and in the prone position was 36% (4/11). In the post-KCOD group, the position last being placed supine was two of two; one of two was found dead in the supine position, and the other was found prone.

Information regarding post-natal exposure to cigarette smoke was available in 11 out of 22 (50%) of post-discharge infants. Of these 11, only 5 (2 SIDS and 3 post-KCOD controls) reported the infant being exposed to cigarettes at home. Information regarding other illicit drugs (i.e., marijuana and methamphetamine) was available on 24 of 32 total KCOD controls and SIDS. In total, only 4 cases (one pre-KCOD control, two post-KCOD controls, and one SIDS) reported any illicit drug use.

## $^{125}$I-Epibatidine Binding Distribution in the Developing Brainstem

We assessed $^{125}$I-epibatidine binding in the medulla and the pons from 26 postconceptional (PC) weeks (midgestation) to 64 PC weeks (∼6 post-natal months) in the pre-KCOD (n = 10) and post-KCOD ($n = 10$) cases for developmental information. The inclusion of pre-KCOD controls allowed us to analyze a greater range of ages and provide increased information on development through gestation. By midgestation binding was

**TABLE 3A |** Smoking through pregnancy and by trimesters.

| | *N* | Mean | STD | Median | Min | Max | Wilcoxin *p*-value |
|---|---|---|---|---|---|---|---|
| **Through pregnancy** | | | | | | | |
| Average cigarettes/week | | | | | | | 0.90 |
| SIDS | 11 | 27.1 | 20.14 | 20.6 | 0.1 | 62.3 | |
| Post-KCOD | 8 | 26.8 | 23.91 | 28.7 | 0 | 58.6 | |
| **By trimester** | | | | | | | |
| **Average cigarettes/week by trimester** | | | | | | | |
| Trimester 1 | | | | | | | 0.54 |
| SIDS | 11 | 26.7 | 20.06 | 23.7 | 0 | 61.7 | |
| Post-KCOD | 8 | 19.2 | 20.32 | 12.1 | 0 | 52.4 | |
| Trimester 2 | | | | | | | 0.90 |
| SIDS | 11 | 27.9 | 21.63 | 22.5 | 0 | 64.4 | |
| Post-KCOD | 8 | 27.7 | 23.80 | 27.6 | 0 | 60.0 | |
| Trimester 3 | | | | | | | 0.72 |
| SIDS | 11 | 26.8 | 19.59 | 21.3 | 0.2 | 60.4 | |
| Post-KCOD | 7 | 27.7 | 31.79 | 7.7 | 0 | 67.6 | |

*N, number; STD, standard deviation; Min, minimum; Max, maximum; Post-KCOD, post-discharge known cause of death; SIDS, sudden infant death syndrome.*

**TABLE 3B |** Drinking exposure through pregnancy and by trimesters.

| | *N* | Mean | STD | Median | Min | Max | Wilcoxin *p*-value |
|---|---|---|---|---|---|---|---|
| **Through pregnancy** | | | | | | | |
| *N* drinks in pregnancy | | | | | | | 0.48 |
| SIDS | 12 | 25.1 | 59.57 | 2.6 | 0 | 210.75 | |
| Post-KCOD | 10 | 25.3 | 35.42 | 8.3 | 0 | 102.5 | |
| **By trimester** | | | | | | | |
| ***N* drinks by trimester** | | | | | | | |
| Trimester 1 | | | | | | | 0.23 |
| SIDS | 12 | 8.3 | 22.80 | 0 | 0 | 80.1 | |
| Post-KCOD | 10 | 10.1 | 13.56 | 3.6 | 0 | 37.5 | |
| Trimester 2 | | | | | | | 0.65 |
| SIDS | 12 | 13.6 | 27.57 | 0 | 0 | 94.8 | |
| Post-KCOD | 10 | 13.5 | 28.27 | 0 | 0 | 69.2 | |
| Trimester 3 | | | | | | | 0.96 |
| SIDS | 12 | 3.3 | 10.31 | 0 | 0 | 35.9 | |
| Post-KCOD | 10 | 1.6 | 4.55 | 0 | 0 | 14.5 | |

*N, number; STD, standard deviation; Min, minimum; Max, maximum; Post-KCOD, post-discharge known cause of death; SIDS, sudden infant death syndrome.*

differentially localized to nuclei of interest in a relatively fixed distribution (**Figure 1**). Medullary binding patterns across all controls combined showed the highest binding in the PIO and lowest binding in the RO (**Table 4**, **Figure 1**). In the rostral pons, binding in nuclei of the pontine tegmentum (MR, LC, and PoO) were relatively high compared to measurements in the basis pontis (GRPO) (**Table 4, Figure 1**). There was a significant developmental decrease in binding with increasing postconceptional age ($p < 0.05$) in the RO, GC, PGCL, and DAO (beta values from −0.39 to −0.25) of the rostral medulla and in the CEN of the mid medulla (beta = −0.22) (**Table 4**). There was a marginally significant developmental decrease binding in in the LC and PoO of the rostral pons ($p = 0.07$; beta = −0.22 and $p = 0.05$; beta = −0.24, respectively). To illustrate this effect, a decrease in $^{125}$I-epibatidine binding with increasing PC age is shown for the RO, GC, and PGCL of the rostral medulla (**Figure 2**). Of note, three rostral medullary sites found to have decreased binding with age (RO, GC, and PGCL) were also found to be significantly affected by nicotine exposure when SIDS and post-KCOD controls were combined (see **Table 6**). The effects of age on these sites remained significant even after adjusting for the effect of smoking (see **Table 6**).

## $^{125}$I-Epibatidine Binding Between All SIDS and All Post-KCOD Controls

Using analysis of covariance (ACOVA) controlling for the effect of PCA, there was a significant effect of diagnosis only in rostral pons PoO ($p = 0.02$) with post-KCOD controls having a higher

**TABLE 4 |** Effect of PCA in Pre- and Post-KCOD controls combined on $^{125}$I-epibatidine binding in brainstem nuclei.

| | PCA-adjusted mean of $^{125}$I-epibatidine binding in specific brainstem nuclei | | Effect of PCA on $^{125}$I-epibatidine binding | |
|---|---|---|---|---|
| | ***N*** | **Mean ± SE (fmol/mg)** | **Beta** | ***p*-value** |
| **Mid medulla** | | | | |
| HG | 20 | 13.03 ± 2.42 | −0.21 | 0.37 |
| DMX | 20 | 13.36 ± 1.69 | 0.19 | 0.26 |
| NTS | 20 | 15.22 ± 2.53 | 0.21 | 0.39 |
| CEN | 20 | 11.82 ± 1.02 | −0.22 | **0.03** |
| PIO | 20 | 23.46 ± 3.08 | 0.27 | 0.37 |
| MAO | 19 | 16.58 ± 1.84 | −0.01 | 0.94 |
| **Rostral medulla** | | | | |
| RO | 20 | 9.35 ± 0.65 | −0.29 | **0.0002** |
| GC | 20 | 13.63 ± 1.15 | −0.39 | **0.002** |
| PGCL | 20 | 11.95 ± 0.81 | −0.30 | **0.001** |
| DAO | 16 | 14.71 ± 1.05 | −0.25 | **0.022** |
| PIO | 20 | 16.82 ± 0.97 | 0.06 | 0.53 |
| **Rostral pons** | | | | |
| MR | 16 | 15.75 ± 1.47 | −0.22 | 0.13 |
| LC | 16 | 13.46 ± 1.20 | −0.22 | *0.07* |
| PoO | 16 | 14.24 ± 1.22 | −0.24 | *0.05* |
| GRPO | 16 | 6.19 ± 0.79 | 0.01 | 0.89 |

*Significant p (< 0.05) are bolded. Marginal p (> 0.05 and <0.1) are in italics.*
*The beta values represent the degree of change in $^{125}$I-epibatidine binding associated with a change in postconceptional age (PCA). Negative beta values indicate a decrease in $^{125}$I-epibatidine binding with PCA. All binding values are in fmol/mg. N, number of cases measured; SE, standard error. Pre-KCOD, predischarge known cause of death; Post-KCOD, post-discharge KCOD; HG, hypoglossal nucleus; DMX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; CEN, centralis; PIO, principal inferior olive; MAO, medial accessory olive; RO, raphe obscurus; GC, gigantocellularis; PGCL, paragigantocellularis lateralis; DAO, dorsal accessory olive; MR, median raphe; LC, locus coeruleus; PoO, nucleus pontis oralis; GRPo, griseum pontis.*

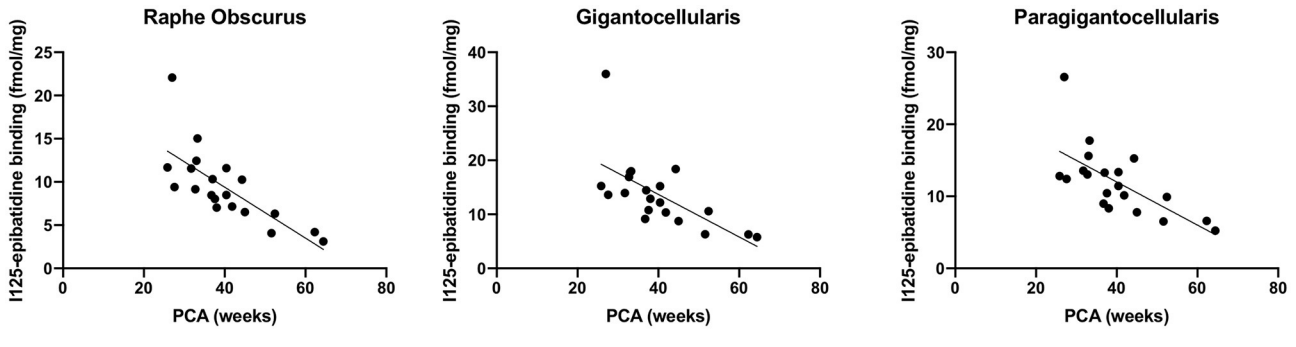


**FIGURE 2 |** Developmental decrease in $^{125}$I-epibatidine binding in the rostral medulla. Combined pre- and post-KCOD controls show a decrease in $^{125}$I-epibatidine binding in nuclei of the rostral medulla. This relationship is illustrated in the RO ($p = 0.0002$), GC ($p = 0.002$), and PGCL ($p = 0.001$).

binding than SIDS (12.1 ± 0.9 fmol/mg and 9.1 ± 0.8 fmol/mg, respectively) (**Table 5**). There was a marginally significant effect in the LC of the rostral pons (p = 0.08) with post-KCOD controls having a higher binding than SIDS (**Table 5**). The demise cohort within PASS has a relatively high prevalence of pre-term birth (58%, SIDS; 50% KCOD) (**Table 2**). Thus, we analyzed $^{125}$I-epibatidine binding in the PASS cohort based on pre-term (<37 gestational weeks) or term birth (>37 gestational weeks). $^{125}$I-Epibatidine binding from pre-term SIDS ($n = 7$) was compared to pre-term post-KCOD controls ($n = 5$), adjusting for PCA. Similarly, $^{125}$I-epibatidine binding from term SIDS ($n = 5$) was compared to term post-KCOD controls ($n = 5$), adjusting for PCA. We found no significant effect of diagnosis in any nuclei in either pre-term or term cases (data not shown).

## Effect of Exposure on $^{125}$I-Epibatidine Binding

We assessed the effect of the amount of maternal drinking (number of drinks consumed) on $^{125}$I-epibatidine binding in all

**TABLE 5 |** Effect of diagnosis controlling for PCA on $^{125}$I-epibatidine binding in the brainstem.

| | Cause of death | | | | | PCA | |
|---|---|---|---|---|---|---|---|
| | SIDS | | Post-KCOD | | | | |
| | *N* | Mean ± SE (fmol/mg) | *N* | Mean ± SE (fmol/mg) | *p*-value | Beta | *p*-value |
| **Mid medulla** | | | | | | | |
| HG | 12 | 8.3 ± 2.34 | 10 | 12.3 ± 2.56 | 0.26 | −0.15 | 0.37 |
| DMX | 12 | 10.8 ± 2.32 | 10 | 14.2 ± 2.53 | 0.33 | 0.16 | 0.33 |
| NTS | 12 | 13.5 ± 3.70 | 10 | 17.0 ± 4.04 | 0.53 | 0.12 | 0.65 |
| CEN | 12 | 9.0 ± 1.26 | 10 | 9.3 ± 1.37 | 0.88 | −0.23 | **0.02** |
| PIO | 12 | 19.8 ± 4.42 | 10 | 27.4 ± 4.83 | 0.27 | −0.21 | 0.51 |
| MAO | 12 | 14.2 ± 3.13 | 10 | 16.1 ± 3.42 | 0.70 | −0.31 | 0.17 |
| **Rostral medulla** | | | | | | | |
| RO | 12 | 6.8 ± 0.80 | 10 | 6.9 ± 0.87 | 0.94 | −0.24 | **<0.001** |
| GC | 12 | 9.9 ± 1.11 | 10 | 10.4 ± 1.21 | 0.73 | −0.30 | **0.001** |
| PGCL | 12 | 9.6 ± 1.08 | 10 | 9.2 ± 1.18 | 0.83 | −0.27 | **0.002** |
| DAO | 9 | 13.6 ± 1.85 | 10 | 12.7 ± 1.67 | 0.72 | −0.35 | **0.011** |
| PIO | 12 | 15.5 ± 1.34 | 10 | 17.9 ± 1.46 | 0.24 | −0.02 | 0.82 |
| **Rostral pons** | | | | | | | |
| MR | 9 | 10.2 ± 1.55 | 7 | 13.0 ± 1.72 | 0.25 | −0.02 | 0.88 |
| LC | 10 | 8.9 ± 0.89 | 7 | 11.5 ± 1.04 | *0.08* | −0.11 | *0.10* |
| PoO | 10 | 9.1 ± 0.78 | 7 | 12.1 ± 0.91 | **0.02** | −0.15 | **0.02** |
| GRPO | 10 | 5.9 ± 0.59 | 7 | 6.5 ± 0.69 | 0.53 | −0.05 | 0.25 |

*Diagnosis p-values are adjusted for postconceptional age (PCA). Means estimated for PCA = 48. Significant p (< 0.05) are bolded. Marginally significant (> 0.05 and <0.10) p-values are in italics. SE, standard error; Post-KCOD, post-discharge known cause of death; SIDS, sudden infant death syndrome; HG, hypoglossal nucleus; DMX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; CEN, centralis; PIO, principal inferior olive; MAO, medial accessory olive; RO, raphe obscurus; GC, gigantocellularis; PGCL, paragigantocellularis lateralis; DAO, dorsal accessory olive; MR, median raphe; LC, locus coeruleus; PoO, nucleus pontis oralis; GRPo, griseum pontis.*

SIDS ($n = 12$) and post-KCOD controls ($n = 10$) combined, adjusting for PCA. There were no significant effects of amount of drinking on $^{125}$I-epibatidine binding in any nuclei (**Table 6**). Given that 50% of SIDS mothers and 70% of post-KCOD mothers reported drinking during pregnancy, we repeated the analyses with exposed-only, SIDS ($n = 6$), and post-KCOD controls ($n = 7$) combined. Similar to the analyses in **Table 5**, the analysis in alcohol exposed-only cases showed no effect of amount of drinking on $^{125}$I-epibatidine binding in any nuclei (data not shown). There was no effect of maternal drinking on pre-KCOD controls (data not shown). Similarly, we assessed the effects of amount of smoking on SIDS and post-KCOD controls combined, adjusting for PCA. A significant positive association between average cigarettes per week and $^{125}$I-epibatidine binding was found in the following rostral medullary nuclei: RO, GC, and PGCL ($p = 0.01$, 0.02, and 0.002, respectively) (**Table 6**). Given the effect of smoking on both SIDS and post-KCOD controls combined, we analyzed each group separately to determine if cigarette smoking had a differential effect on one compared to the other. In post-KCOD controls, we found a significant positive association between $^{125}$I-epibatidine binding and cigarettes smoked per week in the RO ($p = 0.047$) and the PGCL ($p = 0.03$) but little effect in any nuclei in SIDS (**Table 7**). Of note, there was a significant effect of PCA in the RO, GC, and PGCL of post-KCOD controls consistent with the developmental data of **Table 4**. Likewise, there was a significant effect of PCA in these same nuclei in SIDS cases. Given the effect of smoking on $^{125}$I-epibatidine binding in sites of the rostral medulla, we reanalyzed the SIDS vs. post-KCOD control data to examine the effect of diagnosis controlled for smoking and PCA. The significant effect of diagnosis remained in the PoO ($p = 0.04$). A marginal significance of diagnosis was seen in the GC ($p = 0.07$) and DAO ($p = 0.07$) (data not shown).

## DISCUSSION

The Safe Passage Study is the first prospective, multicenter longitudinal study to provide evidence that infants exposed to pre-natal alcohol and cigarette smoke continuing beyond the first trimester have substantially higher risk of SIDS, as compared to those unexposed or exposed only in the first trimester (33). While the first trimester is critical for neurulation and neurogenesis, brain development through the second and third trimesters involves neuronal maturation, synaptogenesis, and synaptic reorganization and pruning—processes that are adversely affected by exposure to nicotine and alcohol (58). Although the fundamental mechanism of pre-natal exposures upon SIDS risk is unknown, we hypothesize that it involves adverse effects on the cholinergic receptor system during brain development. The Safe Passage Study provided an opportunity to examine this hypothesis in an

**TABLE 6 |** Effects of exposure on $^{125}$I-epibatidine binding in the brainstem in SIDS and Post-KCOD controls combined.

| | Alcohol on $^{125}$I-epibatidine | | | | | Smoking on $^{125}$I-epibatidine | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *N* drinks per pregnancy | | PCA | | | Ave. cigarettes per week | | PCA | |
| | *N* | Beta | *p*-value | Beta | *p*-value | *N* | Beta | *p*-value | Beta | *p*-value |
| **Mid medulla** | | | | | | | | | | |
| HG | 22 | −0.01 | 0.76 | −0.17 | 0.32 | 19 | 0.15 | 0.12 | −0.19 | 0.31 |
| DMX | 22 | −0.04 | 0.31 | 0.11 | 0.50 | 19 | −0.04 | 0.56 | 0.15 | 0.27 |
| NTS | 22 | −0.07 | 0.23 | 0.04 | 0.87 | 19 | −0.11 | 0.30 | 0.10 | 0.64 |
| CEN | 22 | −0.02 | 0.28 | −0.25 | **0.008** | 19 | 0.04 | 0.15 | −0.18 | **0.006** |
| PIO | 22 | −0.05 | 0.50 | −0.28 | 0.39 | 19 | 0.08 | 0.46 | 0.08 | 0.70 |
| MAO | 22 | −0.05 | 0.32 | −0.36 | 0.11 | 19 | 0.03 | 0.58 | −0.12 | 0.34 |
| **Rostral medulla** | | | | | | | | | | |
| RO | 22 | −0.01 | 0.45 | −0.25 | **0.0003** | 19 | 0.06 | **0.01** | −0.19 | **0.0001** |
| GC | 22 | −0.02 | 0.22 | −0.32 | **0.0005** | 19 | 0.07 | **0.02** | −0.25 | **0.0002** |
| PGCL | 22 | −0.01 | 0.41 | −0.28 | **0.0014** | 19 | 0.07 | **0.002** | −0.20 | **<0.0001** |
| DAO | 19 | −0.04 | 0.32 | −0.35 | **0.0074** | 16 | 0.03 | 0.39 | −0.20 | **0.007** |
| PIO | 22 | −0.03 | 0.18 | −0.06 | 0.54 | 19 | 0.01 | 0.80 | 0.001 | 1.00 |
| **Rostral pons** | | | | | | | | | | |
| MR | 16 | −0.004 | 0.93 | 0.004 | 0.97 | 15 | −0.06 | 0.32 | 0.03 | 0.82 |
| LC | 17 | 0.01 | 0.85 | −0.12 | 0.12 | 16 | 0.02 | 0.65 | −0.11 | 0.15 |
| PoO | 17 | 0.02 | 0.52 | −0.15 | **0.04** | 16 | 0.03 | 0.45 | −0.16 | **0.04** |
| GRPO | 17 | −0.03 | 0.10 | −0.07 | 0.10 | 16 | 0.02 | 0.42 | −0.08 | *0.09* |

*Significant p (<0.05) are bolded. Marginal p (> 0.05 and <0.1) are in italics. The effects of individual exposure (N drinks per pregnancy or average cigarettes per week) on $^{125}$I-epibatidine binding is adjusted for PCA. N, number; PCA, postconceptional age in weeks, Ave, average; Post-KCOD, post-discharge known cause of death; SIDS, sudden infant death syndrome; HG, hypoglossal nucleus; DMX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; CEN, centralis; PIO, principal inferior olive; MAO, medial accessory olive; RO, raphe obscurus; GC, gigantocellularis; PGCL, paragigantocellularis lateralis; DAO, dorsal accessory olive; MR, median raphe; LC, locus coeruleus; PoO, nucleus pontis oralis; GRPo, griseum pontis.*

international cohort of prospectively collected cases. Our major findings are as follows: (1) there is a developmental decrease in $^{125}$I-epibatidine binding with age in 5 sites within the rostral and mid medulla; (2) there is a decrease in $^{125}$I-epibatidine binding in the PoO, a critical component of the cholinergic ascending arousal system of the rostral pons, in SIDS compared to post-KCOD controls; and (3) smoking affected $^{125}$I-epibatidine binding in 3 rostral medullary sites that contain 5-HT neurons and that have been shown to be abnormal in SIDS infants when examined for the serotonin receptor 1A (5-HT$_{1A}$) (16, 49). These new prospective analyses not only provide reproducibility of developmental changes in cholinergic systems within the brainstem and select cholinergic abnormalities in SIDS but also provide new insights and hypotheses regarding mechanisms by which pre-natal exposures may adversely affect critical medullary neurotransmitter systems involved in cardiorespiratory functions.

## $^{125}$I-Epibatidine Binding in the Brainstem—Effect of PCA on KCOD Controls

The baseline (control) expression pattern of the nicotinic receptors in the brainstem of humans and other species has been studied extensively at the mRNA, protein, and receptor level via the methods of *in situ* hybridization, immunohistochemistry, and receptor-ligand binding autoradiography, respectively [reviewed in Vivekanandarajah et al. (36)]. The relative distribution of $^{125}$I-epibatidine binding in selected human infant brainstem sites of this study is similar in pattern to previous observations with $^{3}$H-epibatidine binding (40), $^{3}$H-nicotine binding (19, 21, 59), and immunohistochemistry (24, 60). In this study, we observed a significant decrease in $^{125}$I-epibatidine binding with increasing PCA in all controls combined (pre- and post-discharge) in the rostral medullary nuclei RO, GC, PGCL, and DAO, mid-medullary nucleus CEN, and a marginally significant effect of age in the PoO of the rostral pons. Given that our KCOD controls ranged from 26 to 64 PC weeks, these data provide information on nAChRs and epibatidine binding prenatally through the second half of gestation and postnatally into the first 6 months of life. The decrease in binding with age suggests that this developmental window is a dynamic period of cholinergic influence on brain development and function. While a decrease in the DAO, PoO, and CEN with age has previously been reported using either $^{3}$H-epibatidine (DAO) (40) or $^{3}$H-nicotine (CEN, PoO) (19), a developmental decrease in the RO, GC, and PGCL using $^{125}$I-epibatidine as a ligand has not been shown. The RO, GC, and PGCL nuclei of the medullary 5-HT system contain 5-HT cells and project diffusely to other regions of the brainstem and the spinal cord. These nuclei form part of the homeostatic network of the rostral medulla, critically involved in cardiorespiratory

**TABLE 7 |** Effects of maternal cigarette smoking on $^{125}$I-epibatidine binding in the brainstem in SIDS and Post-KCOD.

| | Smoking on epibatidine—SIDS Only | | | | | Smoking on epibatidine—Post-KCOD Only | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *N* | Ave. cigarettes per week | | PCA | | *N* | Ave. cigarettes per week | | PCA | |
| | | Beta | *p*-value | Beta | *p*-value | | Beta | *p*-value | Beta | *p*-value |
| **Mid medulla** | | | | | | | | | | |
| HG | 11 | 0.07 | 0.22 | −0.11 | 0.24 | 8 | 0.23 | 0.33 | −0.19 | 0.71 |
| DMX | 11 | −0.06 | 0.50 | 0.13 | 0.38 | 8 | −0.01 | 0.93 | 0.22 | 0.48 |
| NTS | 11 | −0.27 | *0.07* | 0.23 | 0.34 | 8 | 0.03 | 0.87 | 0.01 | 0.98 |
| CEN | 11 | 0.04 | 0.30 | −0.19 | **0.02** | 8 | 0.05 | 0.41 | −0.14 | 0.30 |
| PIO | 11 | −0.08 | 0.21 | 0.22 | *0.07* | 8 | 0.23 | 0.34 | 0.06 | 0.91 |
| MAO | 11 | −0.004 | 0.93 | −0.12 | 0.21 | 8 | 0.08 | 0.55 | −0.02 | 0.95 |
| **Rostral medulla** | | | | | | | | | | |
| RO | 11 | 0.05 | 0.18 | −0.17 | **0.02** | 8 | 0.06 | **0.047** | −0.21 | **0.01** |
| GC | 11 | 0.05 | 0.13 | −0.20 | **0.006** | 8 | 0.09 | 0.10 | −0.31 | **0.03** |
| PGCL | 11 | 0.05 | 0.10 | −0.19 | **0.004** | 8 | 0.09 | **0.03** | −0.19 | **0.04** |
| DAO | 8 | −0.03 | 0.45 | −0.06 | 0.44 | 8 | 0.03 | 0.59 | −0.19 | 0.16 |
| PIO | 11 | −0.11 | *0.10* | 0.14 | 0.20 | 8 | 0.12 | 0.17 | −0.12 | 0.53 |
| **Rostral pons** | | | | | | | | | | |
| MR | 9 | −0.05 | 0.63 | 0.04 | 0.80 | 6 | −0.10 | 0.41 | −0.07 | 0.82 |
| LC | 10 | 0.03 | 0.64 | −0.11 | 0.30 | 6 | −0.01 | 0.85 | −0.16 | 0.31 |
| PoO | 10 | 0.04 | 0.37 | −0.15 | *0.06* | 6 | −0.01 | 0.93 | −0.23 | 0.27 |
| GRPO | 10 | 0.01 | 0.67 | −0.05 | 0.21 | 6 | 0.01 | 0.91 | −0.15 | 0.35 |

*Significant p (< 0.05) are bolded. Marginally significant p (> 0.05 and <0.1) are in italics. PCA, postconceptional age; Ave, average; Post-KCOD, post-discharge known cause of death; SIDS, sudden infant death syndrome; HG, hypoglossal nucleus; DMX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; CEN, centralis; PIO, principal inferior olive; MAO, medial accessory olive; RO, raphe obscurus; GC, gigantocellularis; PGCL, paragigantocellularis lateralis; DAO, dorsal accessory olive; MR, median raphe; LC, locus coeruleus; PoO, nucleus pontis oralis; GRPo, griseum pontis.*

integration and arousal (11). Serotonergic neurons within the nuclei mediate these homeostatic responses (11, 61, 62). Given that serotonergic neurons in these regions express nicotinic receptors (40) and that these serotonergic neurons are likely undergoing developmental changes in 5-HT neurotransmission (as determined by developmental changes in ligand binding to general 5-HT receptor agonist $^{3}$H-LSD), particularly from midgestation to infancy (63), developmental changes in $^{125}$I-epibatidine binding support a dynamic and complex relationship between the neurotransmitter systems—a relationship likely vulnerable to dysregulation during development due to prenatal exposure to nicotine and inappropriate nicotinic receptor binding at these sites. Evidence supporting the effects of nicotine on 5-HT function is detailed below. Studies of additional neurotransmitter systems (including 5-HT) in the PASS cohort are warranted to address potential interrelationships between receptor systems.

## $^{125}$I-Epibatidine Binding Between SIDS and Post-KCOD Controls

In our analysis between SIDS and post-KCOD controls, we found no difference in $^{125}$I-epibatidine binding in the SIDS brainstem in 14 out of 15 brainstem sites. This lack of difference is consistent with the findings of Duncan et al. (21) and Nachmanoff et al. (19), both of which used nicotine as a ligand as opposed to epibatidine and both of which showed no difference in any nuclei when SIDS were compared to controls. We did, however, find a significant decrease in SIDS compared to post-KCOD controls in the PoO and a marginal decrease in the LC, both nuclei of the rostral pons. The LC and PoO are components of the extrathalamic and thalamic arousal system, respectively, and a decrease in cholinergic neurotransmission at these sites in SIDS infants potentially reflect an impairment of arousal responses. With regard to sleeping/waking, the functional significance of a nAChR deficiency in the PoO and LC (marginally decreased) of the SIDS cases compared to controls is unknown. However, given a postulated role of the PoO in the generation of rapid eye movement (REM) sleep (64), we speculate that this lesion may interfere with REM–non-REM (NREM) sleep regulation and the generation of REM sleep in SIDS cases with associated dysfunction in breathing and/or heart rate. Similarly, the LC's involvement in sleep to wake transitions and arousal (65) further support a role for dysfunctional sleep regulation as part of the pathogenesis of SIDS. While the current finding of decreased binding in the PoO and LC in SIDS differs from previous studies that found no change at these sites, these pontine sites were previously identified as affected by maternal smoking (19), thus supporting their inherent vulnerability to alterations in cholinergic development and potentially function.

## Effects of Pre-natal Exposure on $^{125}$I-Epibatidine Binding in the Infant Brainstem

A major finding in this study is that pre-natal smoking is associated with an increase in $^{125}$I-epibatidine binding in SIDS and controls combined in 3 sites of the rostral medulla after controlling for PCA—the GC, RO, and PGCL. Additional analyses show that this significant effect is being driven mainly by post-KCOD controls, particularly in the RO and PGCL where smoking significantly increases $^{125}$I-epibatidine binding in the controls but not in SIDS. Our data of increased receptor binding in response to pre-natal smoking are consistent with the literature (see below) and suggests a compensatory upregulation of $^{125}$I-epibatidine-labeled nAChRs, possibly in response to a decrease in cholinergic activity at these sites (66, 67). Further studies on other cholinergic markers in this dataset are necessary to address the compensatory relationship between cholinergic markers and nAChRs in our dataset. It is of interest that the significant increase in $^{125}$I-epibatidine binding, seen predominately in controls, occurred in three of the five medullary nuclei that are significantly decreasing with age. This represents complex and dynamic neuroplastic changes associated with both development and exposure. The fact that the effect of exposure in the SIDS infants was not statistically significant at these sites suggests a lack of compensatory neuroplasticity within these sites in SIDS. Whether the normal mechanisms shown to be involved in upregulation of nAChRs [post-translational receptor assembly (68), trafficking (68), cell surface expression (68), degradation (68), and affinity alterations (69); post-transcriptional modification by microRNAs (70)] are deficient in SIDS is unknown; however, the potential consequences of incomplete compensation are noteworthy.

Given that acetylcholine modulates serotonergic activity in regulation of cardiorespiratory and homeostatic functions (71–74), an imbalance of acetylcholine transmission (via abnormal nicotinic and/or muscarinic receptors) in SIDS infants likely puts them at risk for sudden death in response to homeostatic challenges. As noted, embedded within the GC, PGCL, and RO are 5-HT neurons. Our data showing an effect of pre-natal smoking in these nuclei support literature reporting an effect of pre-natal nicotine on 5-HT neuron neurotransmission in general (67, 75–77) and directly on medullary 5-HT neurons (78, 79). Numerous experimental and human studies [reviewed in Vivekanandarajah et al. (36)] have shown that maternal cigarette smoke/nicotine exposure adversely affects ventilation (80), breathing drive (81), respiration rate (82–84), ventilatory drive (85, 86), respiratory rhythm pattern generation (87), and arousal responses to adverse stimuli such as hypoxia and hypercapnia (88–91)—responses mediated in part by medullary 5-HT neurons within the RO, GC, and PGCL. It is important to note that maternal smoking exposes a fetus to more than just nicotine, including toxins that have been shown to have unique neurodevelopmental effects and/or combine with nicotine to exacerbate nicotine's effect (66, 67). Given this, our findings are likely not attributed to nicotine alone.

Our data on the amount of pre-natal smoke exposure positively associated with post-natal nicotinic receptor binding are in agreement with the experimental animal models studying the brainstem. Animal models of chronic pre-natal exposure have generally shown increased nAChR expression in the infant brainstem (92–96). Maternal nicotine exposure during pregnancy have resulted in increased nAChR binding in mouse (97) and rhesus monkey (93), increased nicotinic receptor mRNA in the rat (95), and increased nAChR protein expression in the mouse (98) at various brainstem sites. Slotkin et al. also reported an overall increase in $^{125}$I α-bungarotoxin binding in the brainstem that emerged in the early post-natal period (99). Studies of human infant pre-natal exposures demonstrate conflicting results. Our laboratory previously reported an increase in $^{3}$H-nicotine binding in mesopontine nuclei related to cardiorespiration (nucleus parabrachialis) and arousal (LC and PoO) (19) and a decrease in binding in the LC nucleus, periaqueductal gray, and the raphe dorsalis, part of the ascending 5-HT arousal system (21). Other groups have reported decreased α7, β2 nAChR protein expression in the arcuate nucleus, XII, and NTS (22), decreased α4 nAChR fiber staining in the cribriform nucleus (24) in smoke-exposed infants, and increased α7 nAChR protein expression in major brainstem sites associated with pre-natal smoke exposure (100). The differences in the direction of change may be attributable to the composition of the control groups across the datasets with differing cohorts of infants who die from varying causes, the patterns of smoking, and/or the experimental measures, including the use of different ligands and antibodies. Irrespective of direction (up- or downregulation due to exposure), the specificity of change only in KCOD controls is consistent with our other studies showing alterations in controls but not in SIDS. Furthermore, these differences provide an example of the complexity of human autopsy studies where, without measurable cotinine levels, it is unclear whether the differences are due to acute changes in circulating nicotine close to the time of death or due to early or sustained developmental effects of nicotine exposure *in utero*.

Interestingly, there was no effect of the amount of maternal alcohol use on $^{125}$I-epibatidine binding in the brainstem. This differs from the Aberdeen Area Infant Mortality Study (AAIMS) showing a significant reduction in nicotinic receptor binding with an increase in the average number of drinks per month during pregnancy (21). It also differs from experimental studies, both *in vivo* (101–103) and *in vitro* (104), that have shown changes in nicotinic receptor expression following alcohol exposure. It is possible that our continuous measure of drinks per pregnancy is not discerning enough to detect small effects or effects that are trimester specific. It is also possible that the size of the cohort does not have the statistical power to detect effects of alcohol on $^{125}$I-epibatidine. With these caveats, however, our data do support that, in this cohort, pre-natal smoke exposure plays a greater role in altering nicotinic receptor binding than pre-natal alcohol exposure. Although the results of the present study show that amount of pre-natal alcohol exposure does not alter brainstem nicotinic receptors in infants, further studies are warranted to investigate the effect on other neurotransmitter systems, such as the serotonergic system.

### Other Factors of Consideration

Many of the cases (KCOD and SIDS) had some degree of infection, either peripheral or central. In SIDS, this is consistent with minor infection prior to death as a known risk factor (105). The role of nAChRs, specifically the α7 subtype, in neuroinflammation is becoming more appreciated with regard to their function on inflammatory cells (106) and the changes seen in α7 neuronal expression in response to some pathogens and pathogen-specific proteins (107). While we cannot rule out a possible effect of inflammation on our data, given that epibatidine binds the α4β2 nAChR subtype with a much greater affinity than the α7 nAChR subtype (108), an effect of inflammatory-associated influence on α7 nAChR is likely to be minimal. Post-natal exposure to cigarette smoke is another potential consideration (22) as is the effects of pre-natal exposure to cannabinoids (109) from marijuana and methamphetamine (110). Given the relatively small number of cases across SIDS and KCOD controls reportedly exposed postnatally to cigarette smoke ($n = 5$) and prenatally to marijuana and/or methamphetamine ($n = 4$), we did not assess their influence on our data. Larger datasets with quantitative measures of these exposures are necessary to separate out an independent or potentially synergistic influence of these exposures on nAChR binding. Finally, intermittent episodes of hypoxia either prenatally or postnatally have been proposed in the pathogenesis of SIDS (61). While we have no means to identify or quantitate hypoxic events in our cases, the potential influence of hypoxia on nAChR expression should be noted (111–113).

## Limitations of the Study

A major limitation of this study is the relatively small sample size. The validity of results is supported, however, by their general consistency with previously reported data in other cohorts and animal models. A second limitation of this study is the fact that all SIDS, nearly all post-KCOD controls (90%), and all pre-KCOD controls were exposed to pre-natal smoking to some degree, thus making it difficult to detect differences in binding between SIDS and post-KCOD controls controlling for exposure. Despite this, the continuous values measuring pre-natal exposure to smoking enabled us to determine the association between the amount of exposure and nicotinic receptor binding and the developmental trajectory of nicotinic receptor binding with age. A third limitation is that the autopsied control infants were not representative of living controls. This limitation is not specific to the Safe Passage Study but is inherent in all autopsy case/control studies. Another limitation of the study is that there was no experimental verification of the biological concentration of pre-natal exposure in the infant's system. The pre-natal exposures related to smoking and alcohol was based on the mother's self-report of her consumption without verification with biomarkers such as cotinine measurements for smoke exposure and ethanol metabolites for alcohol exposure in infant blood. Finally, the selected radioligand $^{125}$I-epibatidine binds with most, but not optimally, or with all nicotinic receptor subtypes. Additional analysis of and comparison between $^{125}$I-bungarotoxin and $^{3}$H-nicotine would allow for a more comprehensive analysis of nAChRs in the brainstem (114, 115).

## CONCLUSIONS

In summary, our data confirmed our hypothesis that nAChR binding is abnormal in SIDS infants compared to infants dying of known causes. Although significant difference was only detected in one nucleus, the PoO, this abnormality represents a deficit in an arousal system that likely places an infant at risk for SIDS. The fact that deficiencies were only found in one nucleus supports that other neurotransmitters, including 5-HT, may be more affected in SIDS in this database. Relevant to pre-natal exposures, our data support that SIDS infants are not properly responding to or compensating for an effect of pre-natal nicotine exposure by increasing nicotinic receptors. This deficiency, especially in medullary nuclei containing 5-HT neurons, could hinder a normal adaptive/neuroplastic mechanism in SIDS that extends into the post-natal period and decreases the effectiveness of homeostatic responses. Our data showing an effect of pre-natal smoking in medullary nuclei containing 5-HT neurons support the vulnerability of these sites, previously identified as abnormal in SIDS by serotonergic measures, to adverse developmental exposures. The vulnerability is likely heightened during the first year of life when post-natal developmental changes in 5-HT and acetylcholine systems converge. In the Safe Passage Study, we recently reported a synergistic effect of maternal smoking and drinking on SIDS risk—an effect greater than either exposure alone (33). The results reported here showing no effect of maternal drinking on nAChRs suggest that the neuropathological basis for this combined exposure likely involves other systems, including other neurotransmitter systems within the brain. Overall, our results contribute to the wealth of other data, human and animal, supporting the evidence for the adverse effects of pre-natal exposures on a developing fetus, effects that persist into post-natal life, including the period of risk for SIDS.

## DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data is not all publicly available due to regulatory agreements with American Indian tribal nations. Requests for data, however, will be reviewed on a case by case basis. Requests to access the datasets should be directed to robin.haynes@childrens.harvard.edu.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Research Ethics Committee of Stellenbosch University. Other institutional review board approvals, including tribal review boards for reservation-based sites in the Northern Plains, were obtained for all PASS entities (clinical sites, and centers for data coordination, pathology, and physiology). The research was overseen by the Network's Steering Committee

and an external Advisory and Safety Monitoring Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR'S NOTE

The authors of this manuscript from the Safe Passage Study would like to dedicate it to a beloved member of our group, Johan Dempers, who died of COVID-19 days before this manuscript was accepted for publication. Johan Dempers served on the front lines as the Head of Forensic Medicine at the Western Cape Forensic Pathology Service, Tygerberg, and the Faculty of Medicine and Health Sciences of Stellenbosch University in Cape Town, South Africa.

The Safe Passage Study could not have been completed without Johan's leadership and grasp of the impact of infant mortality on his community. He was motivated to pursue SIDS research by a love of children, having two beautiful children of his own. He was passionate about finding the cause of SIDS, especially among disenfranchised groups in South Africa. He was described by many as larger than life, with wide and varied interests, including reading Shakespeare, playing drums in a band (with his daughter as singer and bass guitar) and traveling the world with his beloved family: his wife Karen, daughter Mieneke, and son Daniël. He was proud of his Afrikaans language and heritage and his country, acknowledging its faults of the past to assure a better and more educated tomorrow. He was a wise and enthusiastic scholar and teacher of forensic medicine, a man who loved life and held it sacred in the most difficult settings, including the tragedy of infant death. He took on some of the most challenging, hardened and complex cases in the courtroom with an unshakeable sense of fairness and both academic knowledge and experience to guide the legal profession. He was never without a smile, a word of encouragement, a hearty laugh and the ability to relate to all. His kindness and philanthropy were constant and personal—assisting a friend or stranger just because he could meet a need. We were privileged to know him and to work with him. He became a friend to us all, without exception, and we will greatly miss his joyful and all-inclusive ways.

## AUTHOR CONTRIBUTIONS

RH: had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. HK, AE, WF, MMM, HO, and RH: concept and design. AV, RH, HK, MN, AE, RF, HT, JC, PJ, MM, KM, JRD, KB, KS, HO, JA, LB, EB, JAC, JDD, TB, WF, EG, CG, IH, MMM, BR, PS, MS, CW, DR, LN, DZ, and SW: acquisition, analysis, or interpretation of data. RH, AV, HK, and MN: drafting of the manuscript. RH, AV, HK, MN, AE, HO, WF, MMM, RF, and JRD: critical revision of the manuscript for important intellectual content. MN: statistical analysis. HK, AE, WF, HO, and RH: obtained funding. AV, RH, HK, AE, WF, MMM, HO, RF, HT, JC, PJ, MM, KM, JRD, JA, KB, and KS: administrative, technical, or material support. RH, HK, AE, WF, HO, JA, RF, and CG: supervision. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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The PASS Network is solely responsible for the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript. The following researchers compose the PASS Network:

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.636668/full#supplementary-material

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# A Common 3′UTR Variant of the *PHOX2B* Gene Is Associated With Infant Life-Threatening and Sudden Death Events in the Italian Population

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Heterozygous mutations in the Paired like homeobox 2b (*PHOX2B*) gene are causative of congenital central hypoventilation syndrome (CCHS), a rare monogenic disorder belonging to the family of neurocristopathies and due to a defective development of the autonomic nervous system. Most patients manifest sudden symptoms within 1 year of birth, mainly represented by central apnea and cyanosis episodes. The sudden appearance of hypoxic manifestations in CCHS and their occurrence during sleep resemble two other unexplained perinatal disorders, apparent life-threatening event (ALTE) and sudden and unexpected infant death (SUID), among which the vast majority is represented by sudden infant death syndrome (SIDS). Differently from CCHS, characterized by Mendelian autosomal dominant inheritance, ALTE and SIDS are complex traits, where common genetic variants, together with external factors, may exert an additive effect with symptoms likely manifesting only over a "threshold." Given the similarities observed among the three abovementioned perinatal disorders, in this work, we have analyzed the frequency of *PHOX2B* common variants in two groups of Italian idiopathic ALTE (IALTE) and SUIDs/SIDS patients. Here, we report that the c*161G>A (rs114290493) SNP of the 3′UTR *PHOX2B* (i) became overrepresented in the two sets of patients compared to population matched healthy controls, and (ii) associated with decreased *PHOX2B* gene expression, likely mediated by miR-204, a microRNA already known to bind the 3′UTR of the *PHOX2B* gene. Overall, these results suggest that, at least in the Italian population, the SNP c*161G>A (rs114290493) does contribute, presumably in association with others mutations or polymorphisms, to confer susceptibility to sudden unexplained perinatal life-threatening or fatal disorders by increasing the effect of miR-204 in inducing *PHOX2B* expression down-regulation. However, these are preliminary observations that need to be confirmed on larger cohorts to achieve a clinical relevance.

**Keywords: *PHOX2B*, sudden infant death syndrome, idiopathic apparent life threatening event, miR-204, gene expression regulation, sudden unexpected infant death**

## INTRODUCTION

Paired like homeobox 2b (*PHOX2B*) gene acts in the early development of the autonomic nervous system (ANS), regulating the expression of downstream genes that lead to neuronal differentiation (1). *PHOX2B*, expressed in several districts of the ANS, controls the response to $CO_2$ by integrating chemosensory information derived from carotid body, the nucleus of Tractus Solitarius (NTS), and the Retro Trapezoid Nucleus (RTN) (2, 3). Heterozygous mutations of the *PHOX2B* gene are responsible for congenital central hypoventilation syndrome (CCHS), a rare autosomal dominant genetic condition characterized by impaired response to hypercapnia and hypoxia due to compromised autonomic control of breathing (4–6). In particular, causative mutations are represented by triplet tandem duplications in exon 3 leading to polyalanine (polyAla) expansions, accounting for 90% of CCHS cases, in addition to missense, non-sense, and frameshift mutations responsible for the remaining patients.

CCHS can occur either isolated or in association with other neural crest-derived disorders such as tumors of the sympathetic nervous system (TSNS) and Hirschsprung disease (HSCR), in which not only causative mutations but also *PHOX2B* common variants have been shown to play a role as susceptibility genetic factors (7, 8). CCHS, with the exceptions of rare late onset forms, manifests at birth with central apnea leading to cyanosis, hypercapnia, and desaturation during sleep and can be considered a "chronic" condition persisting lifetime (9). Most patients need to be managed by ventilation only during asleep; however, in a small percentage of cases carrying the largest polyAla expansions, ventilation support is also needed while awake (9).

Interestingly, at symptom onset, CCHS may be viewed as an apparent life-threatening event (ALTE). ALTE is defined as an episode that scares the observer and is characterized by one or more of the following symptoms: (i) apnea (central or occasionally obstructive); (ii) color change (usually cyanotic or pallid but occasionally erythematous or plethoric); (iii) marked change in muscle tone (usually marked limpness); (iv) altered level of responsiveness. This term indicates high-risk conditions leading to the need of resuscitation actions performed by a caregiver, while the more recent term "brief resolved unexplained event" (BRUE) indicates short-lived events that resolve spontaneously and allow patients to be discharged home after a few hours (10). Clinical and/or molecular analyses can disclose a specific etiology in 50–70% ALTE cases, the remaining unresolved events being defined idiopathic ALTE (IALTE) (11).

Previously, these episodes were referred to as "near-miss" sudden infant death syndrome (SIDS) (12). SIDS represents 80% sudden and unexpected infant death (SUID), a fatal event characterized by unexplained death, usually during sleep, of a seemingly healthy baby less than a year old. The incidence of SIDS increases 5-fold in families that had a previous SIDS case (13), thus suggesting that a genetic basis underlies this condition. However, differently from the monogenic CCHS, and according to the so called "triple risk hypothesis," SIDS is regarded to be a complex disorder, in which predisposing genetic factors likely act in concert with external circumstances in a critical developmental period (14). Interestingly, a common variant in the Serotonin Transporter 5-HTT promoter was similarly associated with both SIDS and IALTE (15), thus suggesting a common genetic predisposition in sudden infant death and not clinically explained life-threatening events.

In this light, the early manifestations of CCHS can be seen in ALTE events except that a genetic diagnosis can be achieved later in the former but not in the latter conditions. This has suggested a role of the *PHOX2B* gene in CCHS companion diseases such as SIDS and ALTE. Indeed, a whole PHOX2B gene deletion has been detected in a patient with ALTE (16) and, in different populations, *PHOX2B* common variants have been identified in association with SIDS (17, 18). In addition, brainstem developmental alterations have been identified both in CCHS (19) and in SIDS (20), where *PHOX2B* was shown less expressed and also found in the cytoplasm rather than in the nuclear compartment (21). All these evidences suggest that the etiology of SIDS/SUID, ALTE, and CCHS could share some genetic features among which *PHOX2B* plays a crucial role.

In this manuscript, we are reporting for the first time a genetic screening of the three exons of the *PHOX2B* gene in Italian IALTE and SIDS/SUID cases. A statistically significant association of common *PHOX2B* variants, namely, the rs17885216 (c.552C>T) SNP in exon 3, found in linkage disequilibrium with the rs114290493 (c.*161G>A) SNP in the *PHOX2B* 3′UTR, is reported for the conditions mentioned above. Moreover, *in silico* and functional analyses of possible effects of these two variants were performed.

## METHODS

### Patients Collection and DNA Extraction

Patients have been collected by the SIDS-ALTE Center of Liguria Region (Italy) and the SIDS Center of Meyer Institute (Italy) from 2010 to 2020 (22). Among them, only Italian cases were considered for this work. Informed consent was obtained from patients' parents, and patients' DNA was extracted from either peripheral blood or available autoptic specimens from SUIDs/SIDS, following standard laboratory procedures.

Only individuals lacking any clinical diagnosis, made after the sudden episodes, have been included in the analysis. In particular, 12 IALTE patients and 7 unexpected dead infants, including 6 SUID and 1 SIDS, were considered. Seventy-one DNA control samples, obtained from healthy donors of the Istituto Gaslini and matched for sex but not for age, were also analyzed.

### *PHOX2B* Gene Analysis

The mutation screening of the *PHOX2B* gene (GenBank NM_003924.3) was performed as already reported (6). In particular, the three *PHOX2B* exons were amplified by specific primers (**Supplementary Table 1**) by using the GC Rich PCR System (Roche). Reaction mixes were run for 35 cycles at 95°C denaturation for 1 min, 60°C annealing for 45 s, and 72°C extension for 1 min and 30 s.

PCR fragments were purified with the SapI–ExoIII enzymatic mix by incubating at 37°C for 40′ and at 80°C for 15′ and analyzed for mutations by direct DNA sequencing using the Big

Dye Terminator Cycle Sequencing Kit (Applied Biosystem) on an ABI 3100 DNA automated sequencer.

The *PHOX2B* 7Ala in-frame deletion (hereon 7Ala contraction) was confirmed also by using the "FAM method" (23). In detail, PCR was performed with 22F-FAM 5′-CTGACCCGGACAGCACTGGGGGCC-3′, 5′ end-labeled with FAM, and 279R 5′-GAGCCCAGCCTTGTCCAGG-3′ by the Accuprime GC kit (Life Technologies). Reaction mixes were run for 35 cycles at: 95°C denaturation for 1 min, 62°C annealing for 45 s, and 72°C extension for 45 s, followed by 20 min final extension. One microliter of the PCR product was mixed to 12 μl of formamide and 0.3 μl of ROX 500 size marker (Applied Biosystems) and loaded on the ABI 3100 DNA automated sequencer. Data were then analyzed by GeneMapper (Applied Biosystems).

## Evaluation of the SNPs Allele Phase

DNA samples carrying both SNPs rs17885216 (c.552C>T) in exon 3 and rs114290493 (c.*161G>A) in 3′UTR were amplified with primers: 10F: 5′-TGCTTCACCGTCTCTCCTTCC-3′ and PH2B-3UTR (2) 5′-ATCAGCAGGCGGAGCCC-3′. The product thus obtained, encompassing both loci, was cloned into pCR2.1 (TOPO TA cloning kit, Life Technologies). Colonies were grown in LB/Ampicillin medium, and the plasmids thus obtained, each containing one of the two allele combinations, were sequenced with the primers above to assess the phase of the alleles.

## Statistical Analysis

Fisher's exact test was applied to a 2 × 2 contingency table to evaluate statistical significance of allele frequencies between groups under analysis. Allele frequencies of *PHOX2B* SNPs assessed from a panel of 71 Italian healthy donors were used for comparing cases and controls.

## *In silico* Tools for Predicting *PHOX2B* mRNA Post-transcriptional Modifications

*In silico* prediction of splicing was assessed by using both Alternative Splice Site Predictor (ASSP) (http://wangcomputing.com/assp/) (24) and ESE-Finder 3.0 (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) (25).

For microRNA prediction, MicroRNA Target Prediction Database (miRDB) (http://mirdb.org/custom.html) was used by applying the custom prediction option, while TargetScan 5.2 (http://www.targetscan.org) was used to evaluate conserved sites.

## Construction of c*161G and c*161A (rs114290493) Reporter Plasmids and IMR32 Transfections

A 219-bp region of the 3′UTR *PHOX2B* encompassing the c*161G>A (rs114290493) locus was amplified from a heterozygous SIDS patient. The PCR product was cloned into the pCR2.1 vector (TOPO TA cloning, Life Technologies), sequenced, and, after SacI–XhoI digestion, transferred downstream the firefly *luciferase* gene of the reporter pmirGlo Dual-Luciferase miRNA Target Expression vector (Promega), containing both firefly *luciferase* gene, whose expression is regulated by the exogenous subcloned region, and renilla *luciferase* gene, constitutively expressed and used as internal control.

To evaluate the effect of the rs11429049 c*161 G and A alleles, 150,000 human neuroblastoma IMR32 cells were transfected by Lipofectamine 2000 with 500 ng of pmirGlo plasmids and added at the time of transfection with 50 nM miR-204 mimic or negative control mir(C–) (Dharmacon). After 48 h, Luciferase detection was performed by the Dual Luciferase Reporter System (Promega) with a TD20/20 luminometer (Turner Designs).

# RESULTS

## PHOX2B Screening in IALTE and SIDS/SUID

To search for *PHOX2B* variants that could underlie severe perinatal conditions with, similarly to CCHS, alterations of the autonomous nervous system, the three *PHOX2B* exons have been analyzed in 12 IALTE (7 females and 5 males) patients and a group of 7 unexpected dead infants, including 6 SUID and 1 SIDS (2 females and 5 males). Although no causative variants were identified, we found common variants including a single-nucleotide polymorphism leading to synonymous changes c.552C>T; p.184Ser=(rs17885216) and an in-frame deletion of 7 alanine residues (hereon 7 Ala contraction), shortening the 20 polyalanine (PolyAla) stretch to final 13 Ala residues, in *PHOX2B* exon 3 (**Figure 1**).

In particular, among the four c.552C>T alleles, two were found in IALTE and two were found in the SUID/SIDS group, the 7 Ala contraction was found in an IALTE sample.

To assess a possible difference between minor allele frequencies (MAF) observed in our cases compared to the healthy population, we performed a Fisher's exact test. In particular, an "in-house" panel of 71 DNAs from Italian healthy donors, recruited in the Giannina Gaslini Institute, was used for frequency allele comparison. While the 7 Ala contraction did not differ significantly from the expected frequency, SNP c.552C>T variant allele was statistically more frequent in both IALTE and SIDS/SUID groups compared to controls. The association became even more significant when these two groups were merged (**Table 1**). Of note, by comparing European-non-Finnish allelic frequencies retrieved from the GnomAD v.2.1.1 browser (https://gnomad.broadinstitute.org/), we obtained statistically significant $p$ values for each group (IALTE = 0.029; SUIDs/SIDS = 0.01; IALTE+SUIDs/SIDS = 0.0008).

The c.552C>T SNP was reported in the Ensemble Variant database (http://www.ensembl.org/info/genome/variation/index.html) in complete linkage disequilibrium with a variant in the *PHOX2B* 3′UTR, the c.*161G>A (rs114290493) SNP. Analysis of this latter variant showed that all IALTE and SIDS cases carrying the c.552C>T SNP were heterozygous also for the variant c.*161G>A (26), the two variants sharing in fact the same allelic frequency (0.008) (https://gnomad.broadinstitute.org/region/4-41746099-41750987?dataset=gnomad_r2_1) (27) (**Figure 1** and **Table 1**). The *cis* phase of these two variant alleles was confirmed by subcloning a fragment containing both loci and showing that the two haplotypes included either the


**FIGURE 1 |** Distribution of PHOX2B exon 3 variants in Italian IALTE and SIDS/SUID patients. Graphical representation of the common variants in *PHOX2B* exon 3 identified in ALTE and SIDS/SUID cases. Black line indicates the position of the synonymous variant c.552C>T (p.184Ser=) and c*161G>A, in linkage disequilibrium with c.552C>T; the light gray box represents the 7 polyAla contraction within the 20 polyAla region, shown in dark gray. The TGA stop codon indicates the end of the coding region.

**TABLE 1 |** Minor allele frequency of the *PHOX2B* common variants found in IALTE and SUIDs/SIDSs cases.

| | | **IALTE ($n = 12$)** | | | **SUIDs/SIDS ($n = 7$)** | | | **IALTE+SUIDs/SIDS ($n = 19$)** | | | **Ctrls ($n = 71$)** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Minor allele** | | | **Minor allele** | | | **Minor allele** | | | **Minor allele** | |
| **ID** | **Variants[§]** | ***N*** | ***F*** | ***p*-value** | ***N*** | ***F*** | ***p*-value** | ***N*** | ***F*** | ***p*-value** | ***N*** | ***F*** |
| rs17885216rs114290493 | c.552C>T c.*161G>A | 2 | 0.08 | *0.05** | 2 | 0.14 | *0.02** | 4 | 0.1 | *0.007** | 1 | 0.014 |
| rs746486633 | 7 Ala contraction | 1 | 0.04 | 0.36 | 0 | 0 | na | 1 | 0.03 | 0.51 | 2 | 0.01 |
| TOT/cumulative frequency | 3 | 0.125 | 0.06 | 2 | 0.14 | 0.15 | 5 | 0.13 | *0.02** | 4 | 0.03 | |

[§]*Variant alleles of SNPs c.552C>T and c.*161G>A are in linkage disequilibrium; N, number of minor alleles; F, allele frequency; p-value, asterisks (*) indicate statistically significant values with respect to control samples; na, not assessable.*

reference alleles c.552C-c.*161G (CG) or the variant alleles c.552T-c.*161A (TA). Given the presence of the TA haplotype in IALTE and SIDS DNA samples, we wondered whether its effect could be mediated by gene expression regulation mechanisms able to modify the amount of *PHOX2B* allele product. To this end, we have investigated possible post-transcriptional modifications (PTMs) by using *in silico* tools and performing suitable functional tests.

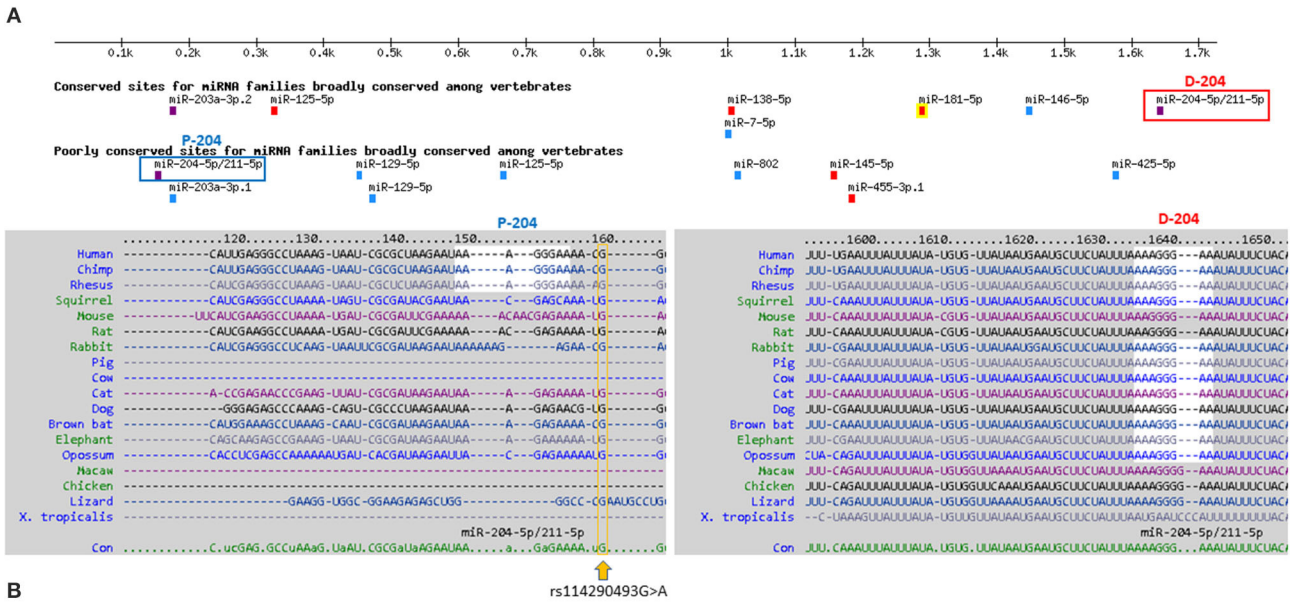
## *In silico* Prediction of the Effects of the c.552C>T and c.*161G>A Variants

As splicing alterations have emerged as a mechanism affecting gene expression regulation (28), we wondered whether the T variant allele of the c.552C>T SNP could alter the exon–exon junctions. Two *in silico* tools, ESE-finder 3.0 and Alternative Splice Site Predictor (ASSP), predicted the T allele to interfere at some extent with the splicing by altering both the 3′ splice site (3SS) and the branch site (**Supplementary Tables 2**, **3**). However, as the c.552C>T SNP is in the third and last *PHOX2B* exon, the "exon trapping" experimental approach usually carried out using the minigene vector pSPL3 (29) turned out to be ineffective to study the splicing, because of the lack of the GT donor site downstream the exon under analysis (data not shown).

We then verified whether *PHOX2B* gene expression could be modified by the common variant c.*161A in the 3′UTR *PHOX2B*, in linkage disequilibrium with the above c.552T variant. In the 3′UTR *PHOX2B*, two sequences are predicted to be bound by miR-204 (**Figure 2A**), one "distal" (D-204), lying within a highly conserved region of the 3′UTR and classified as high conserved miR-204 binding site, the other one "proximal" (P-204), lying within a moderately conserved region of the 3′UTR and classified as low conserved miR-204 binding site (**Figure 2A**). The miR-204-mediated *PHOX2B* expression regulation has already been demonstrated through the D-204 site, together with the effect of the SNP rs1063611 flanking the same D-204 site (26) (**Supplementary Figure 1**). As the SNP c*161G>A is very close to the P-204 site, we checked whether it could display a similar effect. Preliminary *in silico* analysis performed to search for microRNA differently regulating the two alleles showed that, in the presence of the variant c.*161A allele, miR-204 is predicted with a score higher than in presence of the c.*161G allele (**Figure 2B**), thus suggesting that the A allele could induce a reinforced miR-204 mediated down-regulation of the *PHOX2B* gene expression with respect to the G allele.

## *In vitro* Prediction of the c.*161G>A (rs114290493) SNP Consequences

In order to evaluate the effects of the PHOX2B c.*161G>A 3′UTR variant, a 219-bp region amplified from a SIDS patient heterozygous at this locus was cloned in the pmiRGLo reporter plasmid downstream the firefly *luciferase* gene and the construct thus obtained transfected in the IMR32 human neuroblastoma cell line. First, we could observe that this sequence, independently of the c.*161 allele present, induced a decrease in the Luciferase activity with respect to the empty vector, thus suggesting that the sequence cloned was able to drive the down-regulation of the *luciferase* gene expression, thus confirming the suitability of the *in vitro* system (**Figure 3A**). Moreover, the comparison of the miR-204 effect induced on both the G and A constructs showed that addition of miR-204 to the A allele significantly reduced the




| rs114290493 allele | Score hsa-miR-204-5p |
|---|---|
| G | 59 |
| A | 61 |

**FIGURE 2 |** *In silico* prediction of microRNAs binding 3′UTR PHOX2B. **(A)** Target scan release showing microRNA binding the 3′UTR of the *PHOX2B* gene; in the upper part of the figure, the color scale indicates the strength of the prediction, based on the specificity of the seed (violet: 8mer; red: 7mer-m8; cyan: 7mer-A1; green: non-canonical). In the lower part of the figure, the sequences surrounding the proximal, less conserved miR204 site (P-204) and the distal, more conserved miR204 site (D-204) are shown. The c.*161G>A position is boxed in yellow. **(B)** The table shows results obtained by the custom prediction option MicroRNA Target Prediction Database (miRDB); results show the different miR-204 affinity in the presence of the c.*161 G and A alleles. In the two columns, the allele and the score of miR-204 binding are shown.

Luciferase's activity, while it was not able to exert a significant effect on the G allele (**Figure 3B**). To exclude an effect of miR-204 on plasmid backbone, we compared values obtained from the co-transfection of miR-204 with the c.*161 G or A plasmids with values obtained from the co-transfection of miR-204 with the pmiRGlo empty vector. As shown in **Supplementary Figure 2**, overexpression of miR-204 together with the c.*161 plasmids was able to induce a decrease of Luciferase's activity compared to the empty pmiRGlo vector added with the same miRNA, for both the G and A plasmids, the effect on this latter allele being significantly stronger than on the former allele. Both alleles are therefore bound by miR-204, the A allele being more efficient than the G allele. However, when we compared the effects of miR-204 with those of the co-transfection with the negative control miR(C–) on the same c.*161 allele, we observed that the G allele was not modulated by miR-204 while the A allele was confirmed to be down-regulated by miR-204 addition (**Supplementary Figure 2B**). Our results confirmed the hypothesis that the c.*161G>A SNP variant might play a role in *PHOX2B* gene expression regulation mediated by miR-204. Moreover, such an effect was not in contrast with results obtained without miR-204 given that IMR32 cells are characterized by very low miR-204 endogenous levels (30), likely not sufficient to disclose such an allele-specific effect.

## DISCUSSION

In this manuscript we have reported a *PHOX2B* variant screening performed in Italian IALTE and SUID/SIDS patients to search for genetic elements predisposing to these autonomic disorders, whose sudden manifestation may resemble CCHS, caused by *PHOX2B* mutations. While *PHOX2B* has already been analyzed in SIDS (17, 18, 31, 32), it has never been investigated in IALTE so far. Overall, this work represents the first analysis of the *PHOX2B* gene in Italian SIDS and IALTE cases. Results thus obtained showed a statistically significant association between these two groups and the single-nucleotide polymorphism rs17885216 leading to the synonymous nucleotide change c.552C>T in the exon 3 (p.Ser184=). Worldwide, the frequency of the c.552T allele spans from zero (absent) in East Asian, through 0.006718 in Latins, to the highest 0.018 in Africans. For this reason, a matched control population should be used to calculate the statistical significance of the association. This is true also for other *PHOX2B* polymorphisms, such as the intronic IVS2+101A>G (rs28647582) SNP as well as polyAla contractions, which became associated or not associated with SIDS according to the different population ethnicity (17, 18, 32). Similarly, the cumulative frequencies of PHOX2B common variants turned out to be statistically significant in the Caucasian SIDS population but

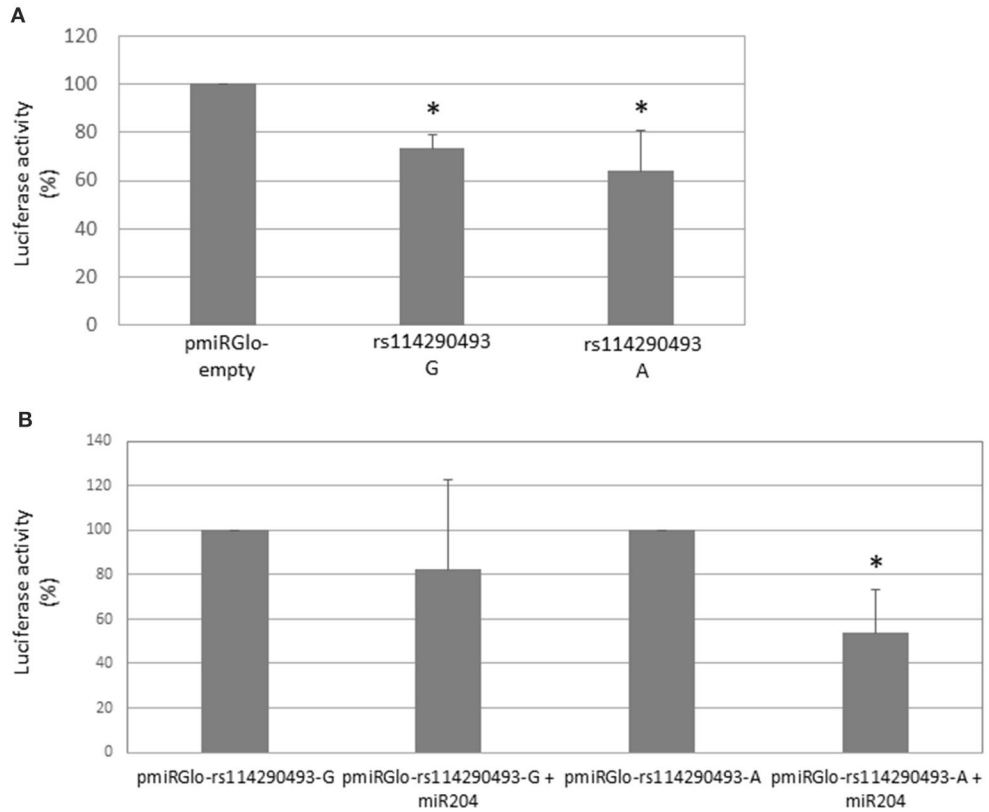



**FIGURE 3 |** Effects of rs114290493 alleles on miR-204 mediated expression modulation. **(A)** The effect of the 219-bp subcloned region on the Luciferase stability is shown in the presence of both G and A alleles; values are expressed as percentage of the empty pmiRGlo vector expressed as 100 and are the mean of three independent experiments performed in triplicate ±SD. Asterisks (*) indicate statistically significant values (Student's *t*-test, $p < 0.05$) obtained by comparing each allele with respect to the empty vector (**p*-value G: 0.015; *p*-value A: 0.019). **(B)** The bar graph shows the Luciferase activity induced by addition of miR-204 as percentage of the value obtained in the absence of miR-204, referred as 100. Values are the mean of three independent experiments performed in triplicate ±SD. Asterisk (*) indicates statistical significance (Student's *t*-test, $p < 0.05$) obtained by comparing each allele added vs. not added with miR-204 (**p* value A: 0.0001).

not in African-American patients (17). Interestingly, *PHOX2B* common variants resulted in a cumulative frequency higher in a set of children affected by obstructive sleep apnea (OSA) with class III malocclusion than in controls (33), an observation that further supports the role of the *PHOX2B* gene in respiratory disorders beyond CCHS, as suggested by OSA occurrence in infants of families with multiple histories of SIDS and ALTE (34). Moreover, the cumulative frequency of *PHOX2B* common variants could reach a statistical significance also in our present Italian IALTE and SUIDS/SIDS cases.

Despite the idea that c.552C>T SNP is predicted to be benign or likely benign by the Ensembl Variation database https://www.ensembl.org/Homo_sapiens/Gene/Variation_Gene/Table?db=core;g=ENSG00000109132;r=4:41744082-41748725, a pathogenic role for this variant was sought. *In silico* prediction did suggest that this variant might impair the *PHOX2B* post-transcriptional regulation through an effect on splicing that however could not be assessed using a standard minigene system.

In addition, to evaluate the c.552C>T change, we have searched also for additional variants that could account for the observed association between common *PHOX2B* variants and Italian SIDS and IALTE cases. The identification of the linkage disequilibrium between the c.552C>T and the c*161G>A SNPs in the *PHOX2B* 3′UTR suggested the following possible scenario: (i) both polymorphisms act in concert to reduce *PHOX2B* expression, (ii) only one of the two is the effective variant while the other is a tag-SNP, and (iii) both SNPs belong to a haplotype where a still unidentified functional variant lies. A functional analysis performed using a Luciferase reporter construct has demonstrated that the c.*161A allele is able to reduce, alone, the *PHOX2B* expression at a higher extent than the c.*161G allele, thus suggesting that this variant induces a loss-of-function effect leading to decreased allele-specific gene expression and therefore to haploinsufficiency. In particular, *in vitro* transfections of the two c.*161 allele reporter constructs did not reveal any effect of miR-204 on the G allele, while the presence of the A allele was always associated with a marked decrease in Luciferase's activity, likely reflecting its role on the entire 3′UTR *PHOX2B*. To perform such studies, human neuroblastoma IMR32 cells were used as expressing high *PHOX2B* levels and already demonstrated suitable to study *PHOX2B* post-transcriptional regulation (26). In particular, they

| | CCHS | ALTE/BRUE | SIDS |
|---|---|---|---|
| **Occurrence** | Mainly during sleep (in most severe cases, also awake) | awake | during sleep |
| **Age of onset** | Most cases < 1 year old but also late onset | < 1 year old | < 1 year old |
| **Outcome** | Not fatal (if promptly managed, but not spontaneously resolvable) | Not fatal (sometimes spontaneously resolvable) | fatal |
| **Inheritance** | monogenic | complex | complex |
| **Duration** | «chronic» (sudden but it lasts lifetime) | sudden | sudden |

**FIGURE 4 |** The scheme represent the clinical and genetic features of the three condition CCHS, ALTE, and SIDS; from left to right, starting from the less severe monogenic CCHS to the fatal complex SIDS, the increase in severity is represented by the color-scale green (CCHS)-yellow (ALTE/BRUE)-red (SIDS).

are characterized by a relatively low miR-204 expression with respect to non-MYCN amplified neuroblastoma cell lines (30), an ideal condition to investigate the effects of this microRNA not only in tumors of the sympathetic nervous system but also in neurodevelopmental conditions. The miR-204 consensus on the PHOX2B 3′UTR was not disrupted by site-directed mutagenesis assays, we could not definitively confirm the miR-204 binding to the proximal site of PHOX2B 3′UTR. Nevertheless, the different effects of miR-204 on the two PHOX2B alleles confirm that, independently of whether directly or indirectly, miR-204 does act also on this proximal site.

Taken together, our present results are in accordance with observations made in neurons from SIDS specimens, where *PHOX2B* expression has been found to be lower than expected (21), thus strengthening the hypothesis of a loss-of-function effect of *PHOX2B* variants in SIDS. The similarity between IALTE and SIDS relies also on the identification of the L/L genotype of the serotonin transporter (5-HTT) polymorphism in both these disorders (15), thus suggesting that they might be different manifestations of a common etiopathogenesis, with SIDS events resembling IALTE episodes occurred during sleep, out of parental control. Therefore, CCHS, ALTE, and SIDS might be members of the same group of respiratory and autonomic disorders of infancy and, along an imaginary severity line, starting from the "chronic" CCHS to the fatal SIDS, they might belong to a same sudden perinatal disorder spectrum (**Figure 4**), with *PHOX2B* variants playing a causative role in CCHS and predisposing to the two other disorders. Taken together, these results confirm that the haplotype including the c.552C>T (rs17885216) and c*161G>A (rs114290493) variant alleles is associated with IALTE and SUID/SIDS. Further genetic tests involving larger cohorts and gene expression analysis on patients' specimens should be performed to confirm that the A allele is able to induce a significant down-regulation of *PHOX2B* expression.

Moreover, as cardiac channelopathies, mainly represented by long QT syndrome (35), are cardiac defects predisposing to sudden death, in further investigations, the role of KCNQ1, KCNH2, and SCN5A genes should be excluded.

Given our homogeneous set of patients, the present genetic data could be valid only in the Italian population. Despite the fact that the role of ethnicity in the variable incidence of SIDS among countries could be due to different socioeconomic environments and caregiving and child-rearing practices, still the virtual absence of the c.552T-c*161A haplotype in the Asian population, where SIDS incidence is very low, and the highest frequency in Africans, characterized by a high SIDS incidence (36, 37), sustain a role of PHOX2B in the etiology of these events. Consistently, the results reported here support the hypothesis of a wider, loss-of-function effect of *PHOX2B* common variants in the predisposition to infant life-threatening and sudden death events.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession numbers can be found below: Leiden Open Variation Database (LOVD), https://databases.lovd.nl/shared/screenings/PHOX2B, Screening IDs: 0000326133, 0000326132, 0000326136, 0000326137, and 0000326134.

## ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

TB designed and performed experiments and wrote the manuscript. SB performed experiments. RP and AP performed clinical analysis of patients. IC data analysis and manuscript editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2021.642735/full#supplementary-material

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# A Rodent Model of Mild Neonatal Hypoxic Ischemic Encephalopathy

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In the brain of full-term newborns, Hypoxic Ischemic Encephalopathy (HIE), a consequence of severe hypoxia and ischemia due to low cardiac output, is frequently observed and results in cerebral injuries with dramatic consequences for life. To investigate the physiopathology of HIE, several animal models have been developed, but none closely replicate human cases, mostly because they are based on a single carotid ligation protocol. In the present study we aimed to develop a novel and more accurate HIE model in juvenile (post-natal days (PND) 14–16) rats. For this, we induced a 9 min hypoxic cardiac arrest (CA) by stopping mechanical ventilation of intubated, ventilated and curarized rats followed by a cardiopulmonary resuscitation. To evaluate the consequences of the CA we performed radiological (cerebral MRI), behavioral (Open Field, Elevated Plus Maze, Fear Conditioning), and histological (Cresyl Violet and Fluoro-Jade B) testing on treated animals. We found that rats in the CA group developed an anxiolytic-like behavioral profile in adulthood without any locomotor impairment, nor memory deficits. However, MRI investigation performed early after CA failed to reveal any change in apparent diffusion coefficient (ADC) in brain tissue (including the hippocampus, striatum, and thalamus), suggesting no massive anatomical lesion had occurred. In contrast, signs of neurodegeneration were found in the Dentate Gyrus and the CA1 region of the hippocampus at day 1 post-CA, suggesting that the anxiolytic-like phenotype observed in adulthood could be related to an abnormal degeneration of this brain region beginning immediately after CA. Thus, our model, despite not representing a severe condition of HIE, nonetheless constitutes a potential model for studying mild, yet persistent and region-specific cerebral injury resulting from an acute oxygen deprivation.

**Keywords: hypoxic ischemic encephalopathy, cardiac arrest, cardiopulmonary resuscitation, development, brain injury**

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## INTRODUCTION

Hypoxic ischemic encephalopathy (HIE) of the full-term newborn remains a significant cause of mortality and morbidity, with an estimated incidence range from 1.3 to 1.7 per 1,000 live birth (1–4). It is the third leading cause of death in children under 5 years of age (3) and can induce neurological disabilities of varying severity (1). In the full-term newborn, HIE causes specific lesions in areas of high energy demand that can differ depending on the degree of cerebral maturation at the time of the insult (5). There are two main types of injury. First, in the context of a moderate decrease in cerebral blood flow, cerebral lesions are mainly cortico-subcortical, para-sagittal (vascular frontier areas) through an antero-posterior vascular redistribution (6, 7) with a milder clinical phenotype outcome, typically without profound motor deficits (8). Second, in

case of a sudden, acute hypoxic event and profound hypotension, adaptive mechanisms fail to develop. In these conditions, the areas of high energy requirement, i.e., the basal ganglia, thalamus, hippocampus, and cortico-spinal tracts, are the most affected (5, 6).

Most HIE rodent models are inspired by that developed by Rice and Vannucci in 1981 (9), itself deriving from Levine's original preparation (10). It involves a unilateral ligation of the common carotid artery followed by hypoxia in 7 post-natal day (PND) old rats, which triggers selective neuronal death or cerebral infarction in the ipsilateral cortex, striatum and/or hippocampus, as well as white matter necrosis. Whereas, this model has been widely used and has led to a better understanding of HIE pathophysiology (11), many improvements have subsequently been proposed, such as a bilateral carotid ligation with or without hypoxia (12), models incorporating reperfusion or models with intra-peritoneal injection of lipopolysaccharide (LPS) to induce an inflammatory response (13). However, these protocols all have major limitations: the focal distribution of cerebral injuries recapitulates more a neonatal stroke injury than the deeper and widespread damage encountered in neonates with a HIE. In 2004, Fink (14) reported an original model of pediatric cardiac arrest, with an 8-min hypoxic cardiovascular arrest resulting in an overall impairment of cerebral blood flow. They found ischemic neuronal lesions in the CA1 region of the hippocampus and in the cortex, as well as persistent memory disorders evaluated by Morris Watermaze. However, this protocol was applied to juvenile P16–P18 rats, which therefore had more advanced brain development than a full-term human newborn (15).

In this context, the aim of our work was to develop an original and practical model that mimics HIE human physiopathology as accurately as possible. We have chosen to adapt the model developed by Fink but making it less invasive, with a prolonged hypoxia duration and applied to younger rats (PND 14–16). In addition, we further assessed our model with MRI analysis, which has not been previously employed.

# METHODS

## Animals

All experiments were conducted under the approval of the Ethics Committee of the University of Bordeaux (referral number: APAFIS#18661-2019012517121887) and the European Union directive 2010/63/EU.

Our experiments used Sprague-Dawley rats ($n = 59$) that were housed with their mother until experimental use or weaning (PND 21). After weaning, they were housed in pairs of same sex, in standard rat cages and with standard nutrient enrichment. The environment was controlled in terms of temperature, hygrometry, and 12/12 h artificial light cycle.

## Experimental Cardiac Arrest Procedure

Mixed-gender PND 14–16 Sprague Dawley rats were anesthetized with 4% isoflurane in an induction chamber ventilated with a 3.5 L/min airflow, until unconsciousness was reached. They were then weighed and placed on an intubation stand (Kent Scientific, Torrington, USA), while the inhaled anesthesia was maintained (airflow 1 L/min, Isoflurane 2%). Rats were then intubated with a 20 Ga angiocatheter, connected to the ventilator SAR-830 A/P (CWE Inc., Phymep, France), set with the following parameters: tidal volume (Vt) 12 ml/kg, respiratory rate (RR) 50/min, insufflation time (Ti) 0.4 s, and fraction of inspired Oxygen ($FiO_2$) 21%. Correct positioning of the endotracheal tube was verified by the observation of a symmetrical thoracic inflation. The animals were then placed on a heating plate controlled by a thermal controller (TC-100 Temperature Controller, CWE Inc., Phymep, France) connected to a rectal temperature probe, with a target temperature of 37°C. Cardiac electrical activity was monitored using three ECG electrodes placed on the limbs and connected to a PowerLab monitor. This monitor was also connected to the ventilator, the thermal controller and a computer to record the following parameters: electrocardiogram (ECG), heart rate (HR), respiratory rate (RR), and temperature. 0.1 mg (2–3 mg/kg) of Rocuronium was administered intravenously (lateral caudal vein) to achieve complete neuromuscular blockade and thus prevent any spontaneous respiratory movement.

In the experimental group, the following cardiac arrest (CA) protocol was applied: animals were exposed to isoflurane for 6 min. The anesthetic was then stopped to allow a 2 min washout period before turning off the ventilator (16). After 8 min of hypoxia, an injection of 5 mcg/kg epinephrine was performed using retro-orbital access (17, 18) in order to reverse cardiac arrest and facilitate resuscitation. The retro-orbital site was used because it was a relatively safe procedure, and standard intravenous injection usually made in the lateral vein was too difficult in such young animals, especially under cardiac arrest. Then, ventilation was resumed after the 9 min of hypoxia using the ventilator controller, with the following parameters: Vt 15 ml/kg, RR 70/min, Ti 0.285 s and FiO2 1. Immediately afterwards, chest compressions were performed at a rate of 200/min. Once per minute, resuscitation was briefly interrupted to observe whether normal sinus activity had resumed. After 10 min of resuscitation, animals were declared dead if there was still no sign of sinus activity, or if resuscitation was successful, the ventilator parameters were gradually changed until the initial parameters were reached, except for FiO2 which was maintained at 1. When effective respiratory movements were observed, the animals were weaned from mechanical ventilation and extubated. They then received a subcutaneous injection of 10 ml/kg saline with 5% glucose to prevent dehydration since they were unable to feed by themselves. After 1 h of observation, they were placed back in their cage with their mother.

Rats in the control group underwent the identical procedure, with the exception of the cardiac arrest stage: they were intubated, curarized, and anesthesia inhalation was continued until 6 min post-injection. A placebo retro-orbital injection (0.9% NaCl) was also administrated.

## MRI Analysis

MRI experiments were performed with a 7 T Brucker BioSpec system (Ettlingen, Germany), equipped with a volume resonator (75.4 mm inner diameter, active length 70 mm) for excitation,

and a 4-element (2 × 2)-phased array surface reception coil. Rats were anesthetized with isoflurane (1.5–2% in air) and positioned with the brain at the center of the Nuclear Magnetic Resonance coil. Animal breathing was monitored during the scanning session (SA Instruments, Stony Brook, NY). A B0 map was drawn with a field of view (FOV) of 30 × 30 × 30 mm. Diffusion Weighted Images (DWI) sequences were acquired with a pulsed gradient spin echo technique, with the following parameters: 50 slices of 0.5 mm thickness, FOV = 25 × 25 mm, matrix = 128 × 128, TE = 24.5 ms, TR = 3,000 ms, six directions of space with b = 1,000 s/mm$^2$; four acquisitions. The acquisition time was 336 s. Anatomical images were obtained using a T2-weighted RARE sequence with the following parameters: 50 slices of 0.5 mm thickness, FOV = 25 × 25 mm, matrix = 256 × 256, TE = 50 ms, TR = 7,818 ms, RARE factor = 8; four acquisitions. The acquisition time was 16 min and 40 s. For each of these sequences, a macroscopic evaluation of injuries was performed on all sections. For each animal, three slices of DWI were chosen, where the following structures were the largest and most visible: striatum, hippocampus and thalamus. Within each of these structures, we measured mean Apparent Coefficient Diffusion (ADC) values (mm²/s) on a 2.4 mm$^2$ square grid. MRI was performed at day 1 after the experimental procedure described above to assess for early injuries (in T2 and DWI) and 7–10 days later to assess for long-term tissue damage on the T2 sequences.

## Histological Analysis

After MRI scanning sessions, the animals were anesthetized with a Ketamine/Xelazine solution and then perfused intracardially and exsanguinated with a peristaltic pump conveying 0.9% NaCl followed by 4% PFA diluted in PBS. After this fixation phase, animals were decapitated, and the brain removed and post-fixed in 4% PFA overnight. Thereafter, the brains were placed for 48 h in a 20% PBS-sucrose solution and then frozen in isopenthane at −43°C to be subsequently sliced (30 or 50 μm thick) using a cryostat (Leica CM 3000).

### Cresyl Violet Staining (19)

Coronal slices of interest were placed on a slide, dehydrated in a bath series with increasing alcohol concentration and then rehydrated in a bath series with decreasing alcohol concentration. They were then placed in a solution of Cresyl Violet for 5 min and then re-dehydrated in the same way. Finally, the slide tissue was cleared by several successive xylene baths. The stained sections were observed and photographed with a Nikon SMZ18 stereomicroscope.

### Fluoro-Jade Labeling and Quantification (20)

Coronal slices (50 μm thick) mounted on gelatin coated slides were first immersed in a solution containing 1% NaOH in 80% alcohol for 5 min. Then slides were successively placed in 70% alcohol for 2 min, distilled water for 2 min and a solution of 0.06% potassium permanganate for 10 min. Slides were then rinsed again in distilled water for 2 min. The staining solution was prepared from a 0.01% stock solution of Fluoro-Jade B (Histo-Chem Inc., Jefferson AR or VWR) with 10 mg of the powder diluted in 100 ml distilled water. This staining solution was prepared freshly for each experiment with 4 ml of the stock solution added to 96 ml of 0.1% acid vehicle. After 20 min exposure to the staining solution, slides were rinsed 3 times in distilled water for 1 min each and thereafter were placed on a slide warmer until they were fully dry. In a final step, slides were cleared by immersion in xylene baths and cover-slipped in a non-aqueous non-fluorescent plastic mounting media (CoverQuick, VWR) (Shumed and Hopkins 2000). Images were acquired with a Zeiss confocal microscope LSM900 and analyzed with the Fiji software. Fluorescence intensities were measured from seven slices obtained from 3 to 4 different animals in the control and the CA groups. Measurements were performed on each half slice from three regions of interest: one region outside the hippocampus where no specific labeling was observed, thus defining the background intensity, one region encompassing the Dentate gyrus (DG), and one encompassing the CA1 part of the hippocampus. The variation of fluorescence was calculated for DG and CA1 relative to background (% ΔF/F).

## Behavioral Experiments

### Open Field (21)

Animals were placed individually for 10 min in a white, low-lit square, and gridded arena with wooden borders and a 102 × 102 cm PVC floor surface, disinfected between each tested rat. A black mark was previously drawn on the heads of the rats with a simple make-up pencil, so that they could be spotted by the video tracking system consisting of a camera placed above the arena and connected to a computer (outside the room), thereby allowing the monitoring of the animal's position. Data were recorded using the ViewPoint software, with integration periods of 60 s. For the analysis, we artificially divided the Open Field into two areas: a central square of 40 × 40 cm and a peripheral area around. Three main measurements were collected: the total distance traveled, the time spent and the distance traveled in each area, and the number of entries into the central area. The latter two parameters are good indicators of anxiety since the central square represents a stressful area for rats that have an aversion to large, open, unfamiliar, and bright environments (22).

### Elevated Cross Maze (ECM) (23)

The rats were placed individually for 5 min in an ECM, 62 cm above the ground, with two arms enclosed by a 40 cm high wall, perpendicular to two open arms, and a transition square between these four arms. The maze was made with dark PVC, measured 116 × 116 cm, and was placed in the middle of a low-lit room. The video tracking system was identical to that used for the Open Field analysis. Data were recorded using ViewPoint software without an integration period. For the analysis, we artificially divided the maze into five areas: one for each arm and one for the central square. The main information collected was the ratio between the time spent in the open arms and the total time spent in all arms (open + closed), noted [OA/(OA+ClA)], which is inversely correlated with anxiety (24).

### Fear Conditioning Test (25, 26)

These experiments were conducted in two stages on consecutive days in 8 identical conditioning chambers (40 × 35 × 30 cm) made of one transparent plastic side and three gray PVC sides. A grid floor (27 metal bars) above a sawdust tray could deliver mild foot electric shocks (0.4 mA AC for 1 s, randomly distributed in arrays of 8 among the 27 metal bars). Stimuli were delivered by a computerized interface (Poly software, Imetronic). Each conditioning chamber was equipped with a miniature (30 × 30 × 32 mm) black and white video camera (SK-2005, OptoVision, Toulouse, France), centered overhead. The camera monitored the entire chamber through a 2.45 mm wide angle lens. Lighting was provided by four LED bulbs through a frosted plastic screen. A set of four cameras was connected to a Quad-type multiplexer (Computar QSMX-II) which combined their four inputs. The resulting video signal was sampled online by a PC type microcomputer equipped with a Scion LG3 video capture card (Scion Corporation, Frederick, Maryland). The principle of the test is to induce Pavlovian associative learning between an environmental context (the illuminated conditioning box) and an aversive stimulus (the electric shock), then to test the persistence of this learning 24 h later. On the 1st day (conditioning phase), the rats were placed individually in a conditioning chamber for a period of 8 min, which was inaugurated by switching on the chamber light. Five electric shocks were delivered at 120, 180, 260, 300, and 400 s. The next day, each rat was returned to the same conditioning box as the day before, but for a longer period (10 min) and without any electrical shock administration. For each animal, we assessed the rate of behavioral "freezing" every minute during both experimental conditions, which was scored manually with a stopwatch. Freezing was defined as the complete absence of movement except that required for respiration, this innate response being a measurement of the fear level in rodents (25, 26).

## Statistical Analysis

The results are presented as means ± SE. For behavioral tests with a dynamic component (Open Field and Fear conditioning test), we used a repeated measures ANOVA. For the other analyses, we applied mean comparison tests. Non-parametric tests were used when the population size was too small, when values were not following a normal distribution, or when there was no homogeneity of variance between groups. Note that due to an insufficient number of male rats having undergone a control procedure, we had to use a heterogeneous control group related to the inclusion of four healthy rats out of six. All statistical analyses were performed with R software, with a probability threshold for rejection of the null hypothesis ($p$) of 0.05.

# RESULTS

## Experimental Cardiac Arrest Procedure

Fifty-nine animals were included in our study, with 48 comprising the cardiac arrest (CA) group and 11 in the control group (6 rats that died from tracheal injury following intubation difficulties during the first procedures were not considered). The experimental flowchart for the entire study is presented in **Figure 1**. In the CA group, mortality was quite high (41.67%), due to resuscitation failure from persistent atrioventricular dissociation ($n = 14$) and deaths within 24 h of effective resuscitation ($n = 6$). However, note that the survival rate, ranging around 58% in the CA group, increased over time to approach 80% during the last series of experiments, mainly due to a significant progressive improvement in our technical skills in manipulating such young animals. Thus, when mastered this protocol of cardiac arrest followed by resuscitation is reproducible and provide to be a good tool to investigate pathological consequences of CA. The total protocol duration (from curare injection to extubation) was 58.4 ± 16.2 min in the CA group and 33.8 ± 5.5 min in the control group ($p < 0.001$). This significant difference was due to several longer protocol phases for the CA group (resuscitation time and a slower recovery of spontaneous breathing). When comparing the CA and the control group, there were no statistically significant differences in weight, sex, and age (**Table 1**). In the CA group, the mean duration of bradycardia (HR < 120/min) was 420.8 ± 49.4 s. Prior to resuscitation, most animals showed a pattern of extreme bradycardia (HR < 60/min) or asystole; no expression of ventricular tachycardia or ventricular fibrillation was recorded. In the control group, HR remained stable throughout the procedure (370 ± 40/min).

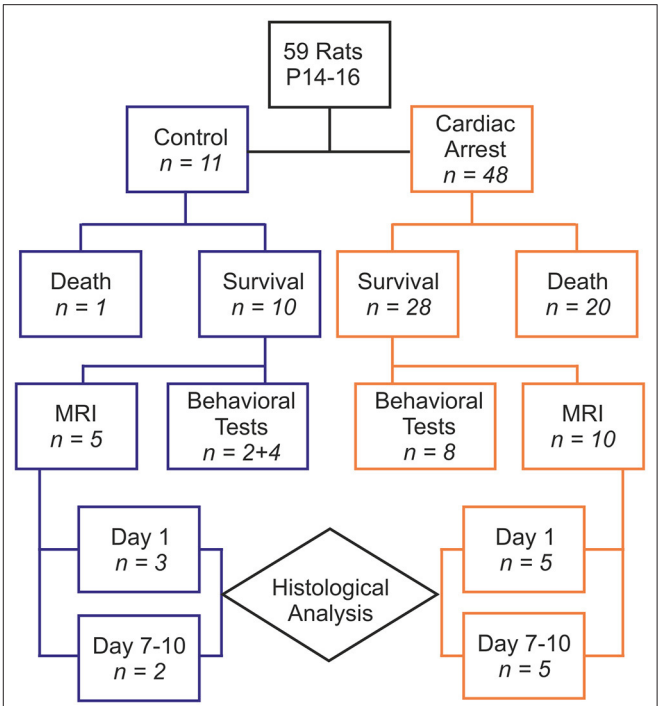


**FIGURE 1 |** Diagram showing the experimental paradigm of the study with the group distributions and numbers of animals designated to each test performed.

## MRI Analysis

Following the CA procedure, MRI observations were conducted on these animals at two distinct times: 1 day following the CA to

**TABLE 1 |** Characteristics of animals included in the study.

| Characteristic | CA ($n = 28$) | Control ($n = 10$) | Stats | $p$ |
|---|---|---|---|---|
| **Characteristics by group** | | | | |
| Mean weight (gm) | 37.37 ± 6.41 | 35.20 ± 4.66 | $t = 0.98$ | 0.34 |
| Sex ratio (M/F) | 3.50 | 1.00 | $\chi^2 = 2.70$ | 0.10 |
| Age (PND) | 14.96 ± 1.11 | 14.60 ± 0.52 | U = 125.00 | 0.61 |

**TABLE 2 |** ADC values at day 1 and days 7–10 after experimental procedure.

| Structure (Day post-procedure) | CA | Control | Stats (U) | $p$ |
|---|---|---|---|---|
| **Mean ADC value ($10^{-4}$ mm$^2$/s) by group** | | | | |
| Hippocampus (D1) | 7.73 ± 0.95 | 8.36 ± 0.82 | 6.00 | 0.79 |
| Striatum (D1) | 8.17 ± 0.43 | 7.49 ± 0.57 | 1.00 | 0.67 |
| Thalamus (D1) | 7.86 ± 0.56 | 7.99 ± 0.72 | 4.00 | 1.00 |
| Hippocampus (D7–10) | 8.48 ± 1.09 | 8.65 ± 0.89 | 4.00 | 1.00 |
| Striatum (D7–10) | 8.66 ± 0.93 | 8.02 ± 1.32 | 3.00 | 0.80 |
| Thalamus (D7–10) | 8.40 ± 1.09 | 7.97 ± 1.01 | 3.00 | 0.80 |

*ADC, Apparent Diffusion Coefficient; D1, day 1; CA, Cardiac Arrest.*

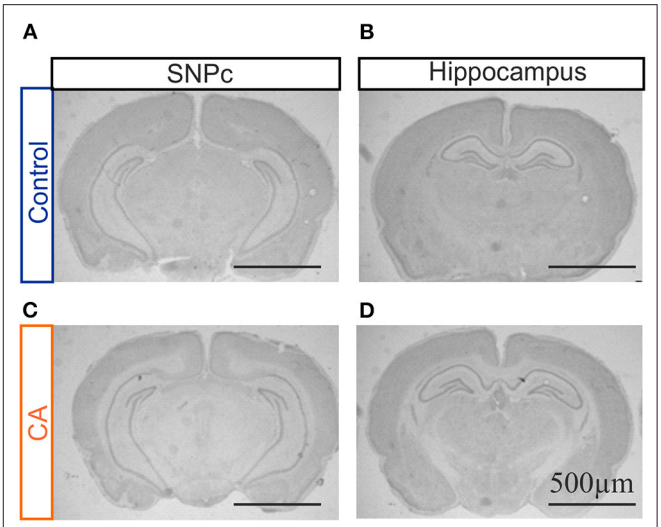


**FIGURE 2 |** Photographs of coronal brain slices obtained from a control rat **(A,B)** and a rat previously subjected to a cardiac arrest (CA) **(C,D)** at the level of the substantia nigra pars compacta (SNPc; **A,C**) and the hippocampal region **(B,D)**. Slices were stained with cresyl violet. Note the absence of any major anatomical damage in the CA brain.

investigate potential acute effects of CA, and 7–10 days later to evaluate more slowly developing cerebral damages. We focused on brain structures known to be affected by HIE in humans, such as the hippocampus, striatum and thalamus. In the CA group compared to the control group, we did not observe any massive anatomical lesions in conventional T2-weighted imaging (data not shown). Furthermore, no significant differences between the groups regarding mean ADC values in the three structures studied were evident (**Table 2**), whatever the period post-CA. These results thus indicated that no major anatomical anomalies were detectable in CA-subjected animals using MRI. However, it remains possible that less pronounced anatomical deficits might exist but could not be revealed by MRI, especially due to the small size of the animals used in the present study.

## Histological Analysis

In order to complement our MRI assessment and to proceed to a finer characterization of potential anatomical lesions in brain tissue, we conducted a qualitative histological analysis with Cresyl Violet staining on brain slices obtained from animals having been subjected to the CA paradigm and compared these to the equivalent tissue regions from control animals. A careful observation was made of all brain structures, but here again with a specific interest in the hippocampus, striatum and thalamus. However, when compared to control brains, slices from CA animals showed a typical anatomy, as illustrated for the hippocampus in **Figure 2**. Similar results were obtained for tissue harvested 1 day after the CA and at 7–10 post-trauma, indicating (as from MRI) that significant brain lesions resulting from a CA, if any, do not emerge in the 10 day time period following the injury or are very spatially limited.

In a second series of experiments, we sought signs of neuronal degeneration in brain tissue following the acute CA procedure. To this end, Fluoro-Jade B staining was used to identify such a process. A greater Fluoro-Jade B staining in the Dentate gyrus (DG) and the CA1 region of the hippocampus was found in rats from the CA group compared to these same brain regions in the control group when brain sampling was performed at day 1 after the CA procedure ($n = 14$ measurements from 7 slices obtained from 3/4 different animals in each group; **Figure 3**). We found an intensity of fluorescence that was 35 ± 4.7% significantly ($p < 0.001$) higher in the CA1 of the CA group compare to control slices, and 19 ± 5.9 % significantly ($p = 0.02$) higher in the DG in CA group compare to control slices (**Figure 3C**). In contrast, inspection of other brain areas did not reveal any signs of abnormal neuronal death. Thus, consistent with other results of our study (see below), our anatomical investigation of brain tissue failed to reveal any widespread, evident anatomical defaults in rats having previously suffered from a 9 min CA, although there were early signs of region-specific neurodegeneration, localized to the hippocampus.

## Behavioral Analysis

In a further exploratory approach, we performed a series of behavioral measurements to test for functional correlates of this hippocampal neurodegeneration. The main parameters evaluated were motor function, anxiety and a form of hippocampal-dependent memory. These tests were conducted on adult male rats (PND 56–80) in order to assess for stable and long-lasting deficits resulting from the 9 min cardiac arrest applied previously at PND 14–16.

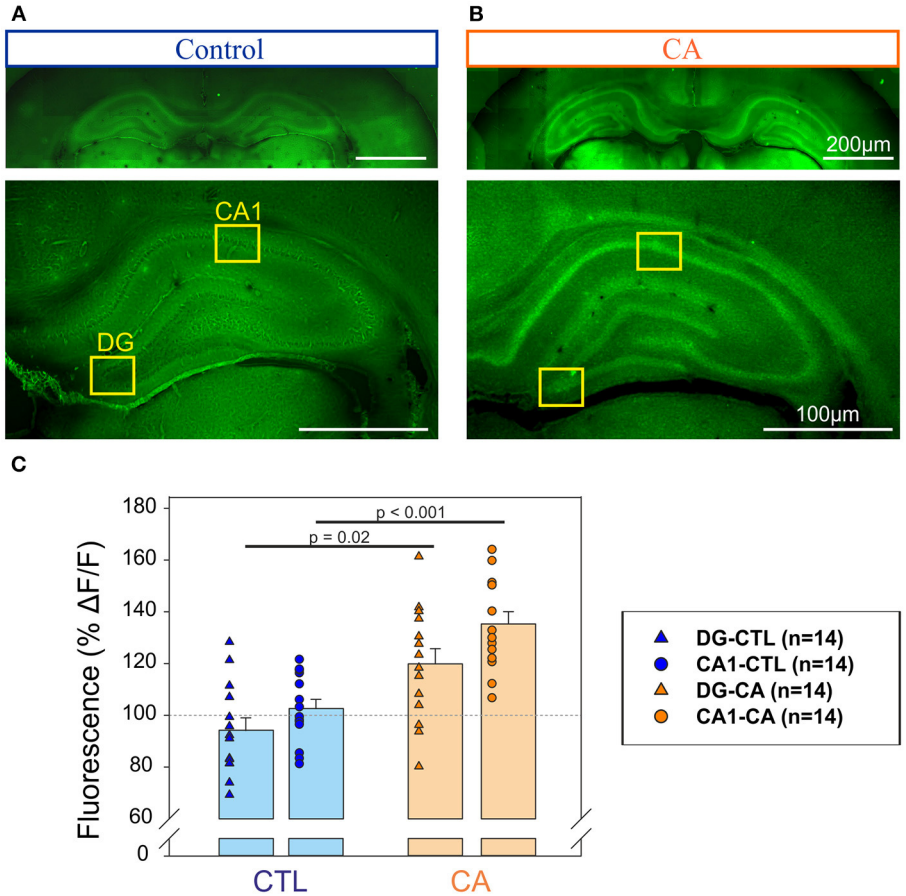


**FIGURE 3 |** Detection of neurodegeneration in the hippocampus with Fluoro-Jade. Photographs of coronal slices obtained for control animals **(A)** and animals exposed to a CA at Day 1 **(B)** at two different magnifications (top, bottom). Note that in each case top and bottom images have been obtained from different slices. The yellow rectangles delineate the Dentate gyrus (DG) and the CA1 region of the hippocampus in which fluorescent measurements have been performed to be compared to background signal. **(C)** Histograms representing the % of relative change of fluorescence (ΔF/F) in the regions of interest (DG, triangles and CA1, circles) for control ($n = 14$, blue symbols) and CA1 ($n = 14$, orange symbols). A significantly stronger intensity of fluorescence in the DG and CA1 regions of the hippocampus in the CA tissue, suggesting a higher degree of neuronal degeneration.

## Open Field

In both CA and control groups, locomotor activity in the open field gradually decreased over time with similar trajectories, with both the total distance covered (**Figure 4A**) and the distance covered near the area edges (**Figure 4B**) decreasing significantly ($F_{(9.108)} = 6.24$, $p < 0.01$ and $F_{(9.108)} = 14.18$, $p < 0.01$, respectively). This suggests the development of an habituation phenomenon that was apparently similar in the two experimental groups (absence of Time*Group effect: $F_{(9.108)} = 0.29$, $p = 0.98$). Consequently, locomotor activity was comparable in both groups: there were no significant inter-group difference either in total spontaneous displacement ($F_{(1.12)} = 4.05$, $p$=0.07) or in displacement close to perimeter edges ($F_{(1.12)} = 0.99$, $p = 0.34$).

In contrast, rats in the CA group spent significantly more time in the central square (**Figure 4C**) than control group animals ($F_{(1.12)} = 13.49$, $p < 0.01$), and they were more likely to explore the central square as indicated by the consistently higher distances that they traveled whilst in this more exposed area ($F_{(1.12)} = 6.51$, $p = 0.03$; **Figure 4D**). It is noteworthy that although this latter difference was not sufficiently elevated to convey mean values for total distance covered (**Figure 4A**) into a range that was significantly different from control ($p = 0.07$), it very likely participated in the trend observed. Furthermore, for all of these parameters, the "Time" variable had no significant effect. There is also no statistically significant Time*Group interaction, although there was an evident trend toward the development of such a group effect in both the amount of time spent, and the distance traveled, within the central square ($F_{(9.108)} = 1.79$, $p = 0.08$). Taken together, therefore, these results strongly suggest that rats in the CA group exhibited a level of behavioral anxiety that was lower than that of their non-CA counterparts.

## ECM Analysis

In the elevated cross maze test, the proportion of time that rats spent in the open arms (i.e., the [OA/(OA+ClA)] ratio) was significantly higher in the CA group than in the control group (64 ± 12 vs. 46 ± 14%, U = 6.00, $p = 0.02$) (**Figure 5A**). No significant difference was observed between the CA and control

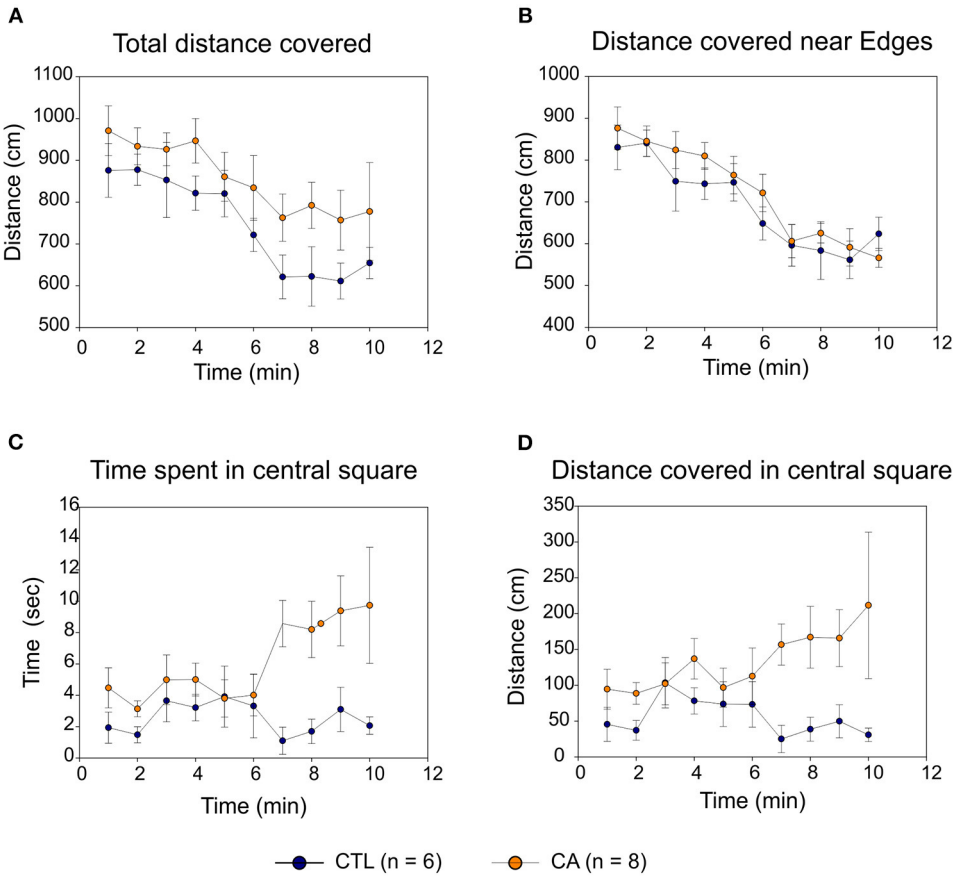


**FIGURE 4 |** Open Field behavioral testing. The graphs indicate distances covered in the open field over time **(A)**; distances covered near the field perimeters **(B)**; times spent in the central square **(C)** and distances covered in the central square **(D)** for the control group (CTL; blue symbols) and the cardiac arrest (CA) group (orange symbols). Values are expressed as Mean ± SE.

groups regarding the total time spent in both open and closed arms of the maze (166 ± 32.4 vs. 160 ± 18.2 s, U = 18.00, $p = 0.49$; **Figure 5B**). This indicates that rats in the CA group are more likely to explore open arms than rats in the control group, although this is not due to greater locomotor activity. These findings are therefore consistent with those obtained from the Open Field analysis described above, further supporting the conclusion that a CA leads to a decrease in physiologic unconditioned fear responses.

### Fear Conditioning Test

In a final set of behavioral experiments, we investigated potential memory impairment resulting from a 9 min CA. For this, we used a classical fear conditioning test involving the behavioral freezing response (see section Materials and Methods). During the conditioning phase, the freezing rate increased significantly over time ($F_{(7.84)} = 73.03$, $p < 0.01$), but without any significant difference between groups ($F_{(1.12)} = 0.04$, $p = 0.84$), nor was there a significant Time*Group effect ($F_{(7.84)} = 0.77$, $p = 0.62$) observed. Thus, animals in both groups appeared to gradually develop a conditioned fear response in a comparable way. Subsequently, during the recall phase, we observed a significant decrease in the freezing rate over time ($F_{(9,108)} = 12.86$, $p < 0.01$), and again, there was no significant difference between the CA and control groups ($F_{(1,12)} = 0.90$, $p = 0.36$) (**Figure 6**). There was also no interaction between time and group variables ($F_{(9.108)} = 1.53$, $p = 0.15$). Thus, altogether these results do not support the possibility of a long-term memory impairment in animals subjected to a CA.

## DISCUSSION

In this study, we have successfully developed a low-invasive protocol for an acute (9 min) hypoxic cardiorespiratory arrest in PND 14–16 rats. We demonstrated that this protocol leads specifically to a neurodegeneration in the CA1 region of the hippocampus at day 1 after CA, an effect that is associated with the later expression of anxiolytic-like behavior at adult stages but without any loss in memory capabilities. However, the use of MRI for gross anatomy assessment and cresyl violet staining for finer observations failed to reveal any significant anatomical defects that could underlie this behavioral change. Nevertheless, our data indicated that a short-lasting cardiac

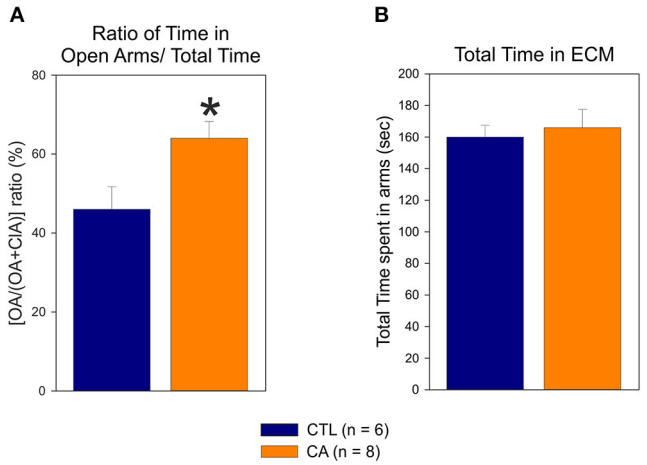


**FIGURE 5 |** Elevated Cross Maze test: the histograms represent the ratio of time spent in the open arms (OA) relative the total time spent in the maze **(A)** and the total time spent in both open and closed arms (CIA) **(B)**. Values are means ± SE obtained from 6 control (CTL, blue bar) and 8 cardiac arrest (CA, orange bar) animals. Asterisk indicates significant difference ($p < 0.05$, Mann Whitney test).

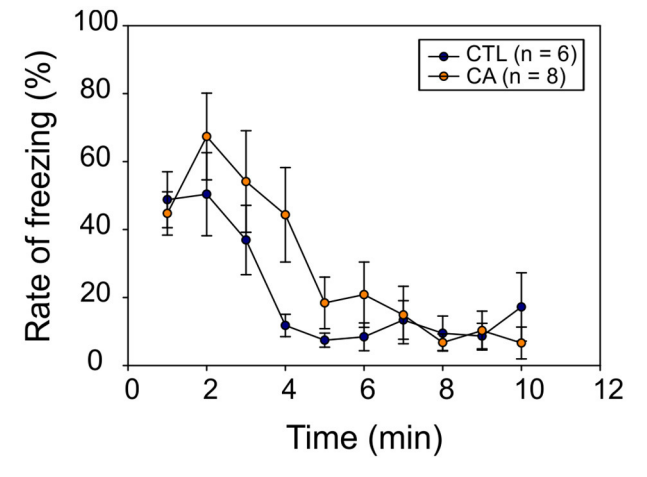


**FIGURE 6 |** Recall phase of the fear conditioning test. The graph represents the mean behavioral freezing rate (in %) over time obtained for 6 control (CTL, blue symbols) and 8 CA animals (orange symbols). Data are expressed as Mean ± SE.

arrest triggers abnormal neuronal degeneration, at least in the hippocampus, that subsequently becomes discernible a few days later. Thus, our model could be useful to better understand the neurophysiological consequences of an HIE occurring at young ages.

## Behavioral Changes Induced by HIE

Rats that had undergone our experimental CA procedure were found to develop reduced levels of unconditioned fear responses (anxiolytic-like behavior) in adulthood, as evidenced by both Open Field and ECM assessment. Similar results were obtained by Wang et al. following an intracerebral injection of LPS in PND 5 rats (27), where atrophy and neuronal death in the CA1 region leading to adult memory deficits were not only observed, but also less anxiety-like (anxiolytic-like) responses in the ECM task. This could be explained by lesions to the ventral hippocampus, since this structure also plays a role in the regulation of emotional behavior (28). Indeed, lesions of the septo-hippocampal system, which is the neural substrate of behavioral inhibition, may lead to a decrease in fear and anxiety reactions (29, 30). However, the consequences of hippocampal lesions for anxiety and fear behaviors remains unclear since other studies have reported the expression of hyper-anxious behavior after prenatal hypoxia-ischemia (31) or after neonatal hippocampal lesions in non-human primates (32). Therefore, additional experiments are required to unequivocally associate defaults in the hippocampus with this anxiolytic-like behavior in the context of our paradigm used to induce HIE. Notably, we could use tests that would allow us to discriminate this anxiolytic-like behavior from depression, but the latter is probably not importantly developed here as the general locomotor behavior is normal and not reduced as expected in the case of depression. In addition, it could be relevant to measure plasma corticosterone levels to characterize neuroendocrine stress responses during a stressful procedure, in order to quantitatively assess the anxiolytic-like behavior shown by CA rats.

Furthermore, in our case, we did not find any significant memory deficits in rats of the CA group, although this conclusion thus far derives from a single test (Fear conditioning test) and should be complemented with experiments using other memory tests, such as with Morris water maze procedures. Interestingly, it has been shown that a selective lesion of the ventral hippocampus in rats is able to induce a decrease in unconditioned fear responses, as well as decreased neuroendocrine stress responses, but without impairing contextual fear conditioning nor spatial navigation (33).

In addition, we did not observe locomotor impairment in rats of the CA experienced animal. However, although a model of severe HIE would be expected to induce significant motor impairment, as observed in neonates (1), this outcome is rarely found even in studies using the Vannucci protocol at PND 7 (34). Accordingly, with our model that evidently produces a mild HIE, associated locomotor deficits might also be relatively moderate and difficult to pin-point without using more specific tests.

Nevertheless, attempting to extrapolate the results from our animal model to the consequences of HIE in humans requires caution when interpreting the severity of a neurological or behavioral deficit. For instance, it is possible that abnormally low unconditioned fear responses, a physiologic defensive behavior, might effectively place a rat at far greater risk than it would in a human. In other words, this anxiolytic-like state in a rat might expose it to a level of vulnerability that in humans is more equivalent to the vulnerability arising, for example, from a hemiparesis that often results from a severe HIE.

## Anatomical Damage Caused by HIE

Following a 9 min hypoxic cardiorespiratory arrest, we expected to detect important anatomical signs of damage to brain tissue.

However, MRI inspection performed either in the acute phase (day 1) or after more than a week (days 7–10) failed to reveal any obvious structural alterations, although several methodological factors might be relevant to our employment of this technique. First, the use of isoflurane as an anesthetic could modify cerebral blood flow and then may perturb the consequences of HIE in rats (neuroprotection) (35). Second, the delay chosen between the application of the CA procedure and MRI assessment may not have been the most appropriate. In a first instance, these delays were selected in analogy to the human pathology, but they probably would require adjustment in future investigations. In humans when using MRI, cerebral hypoxic ischemic damage can be visualized by a T1 hyperintense signal appearing between 3 and 5 days following a T2 hypointense signal occurring between postnatal days 6 and 10. Later, depending on the severity of the lesion, atrophy of the basal ganglia and thalamus, persistent cortical T2 hypersignals, thinning of the corpus collosum, and sometimes cavitation or even multi cystic encephalopathy can be observed (5). During the first 2–3 days of life, however, these structures can appear normal under MRI. However, DWI sequences are able to detect lesions in the acute phase (36) that are closely correlated with histopathological data (37), but with two limitations: an underestimation of the lesions if performed too early (<24 h) and a sensitivity that declines after 7 days (5). Third, T1-weighted imaging could also have been performed in addition to T2 imaging, since subacute injuries (before 5 days after a CA in the human newborn) are not visible in the latter sequences. Finally, we cannot rule out the possibility that investigating brain anatomy of such small rodents using MRI might be confronted with an insufficient spatial resolution that prevents detection of fine tissue damage.

To try to circumvent this potential problem we complemented our first set of MRI observations with a series of experiments using standard cresyl violet histological staining of thin brain slices. Here again, however, no obvious signs of anatomical lesions in different brain structures could be detected following our HIE protocol. One possibility is that either our CA procedure was not sufficiently disruptive to induce significant tissue lesions, or that once again the post-CA timing of our anatomical observations was inappropriate: making observations after only 1 day might have been too premature for processes of tissue necrosis to have occurred or be sufficiently advanced to become detectable. Thus, in the case of such limited alterations, a densitometric analysis and/or a more precise neuronal cell count could have provided a finer anatomical assessment that revealed milder tissue lesions. In any case, if our model actually induces brain lesions, they are mild at most. Usually, tissue resistance to hypoxia varies across species (38). Therefore, it is possible that rats have a better cerebral tolerance to hypoxia than humans (39), which could explain our incapability to reveal severe lesions after a protocol of profound hypoxia such as ours.

Nevertheless, our results do suggest that our model of HIE induces hippocampal damage. Specifically, Fluoro-Jade labeling of neurons in the CA1 region of this structure, indicating the occurrence of abnormal neurodegenerative processes (20). Importantly, such a specific structural loss could underlie the behavioral changes observed in our animals after brain oxygen deprivation (see above).

## Validity of Our Model and Perspectives of Improvement

Our model differs in several ways from that developed by Fink et al. (14). First, in our study we used younger rats, PND 14–16. Alternatively, the ideal age for an HIE model is in fact at younger PND 7–13, since cerebral development in this stage range is comparable to that of a full-term newborn in terms of myelogenesis and the expression oligodendrocyte maturation markers (15, 40). In a preliminary set of experiments, we attempted to use such younger rats but were confronted with a high degree of mortality, mainly due to the difficulty of intubating animals at these ages. Thus, considering our aim to mimic human neonatal hypoxic CA and resuscitation as closely as possible from a physiopathological perspective, we used the youngest possible rats; although we are aware that this age range is not ideal for a neonatal HIE model. Nevertheless, during the course of our study the survival rate increased over time, reflecting the improvement of our protocol and of our intubation technical skills. Applying our experimental procedure to younger PND 12–13 rats to continue developing and validating our animal model for HIE is therefore conceivable for future experiments.

Furthermore, compared to the Fink model we chose a longer hypoxia duration (9 min instead of 8 in the Fink model) in order to increase the probability of neurological lesions. Our preliminary investigations with a 10 min CA duration resulted in a high mortality rate. It therefore seems impractical to further increase hypoxia duration with our protocol, although the problem could be alleviated by modifying some parameters of our experimental procedure, such as by applying moderate hypoxia before switching off the ventilator.

In addition, our model is much less invasive than Fink's model, which could constitute a strength as well as a weakness. On one hand, by avoiding surgery, we reduce the duration of the protocol, the duration of exposure to isoflurane, risk of infection, and blood spoliation. On the other hand, however, without catheterization, resuscitation must be performed "blindly": our ventilatory parameters are set up arbitrarily since we cannot perform arterial blood gas analysis; and without invasive blood pressure measures we only have an indirect indication of the occurrence of a cardiac arrest, which is unreliable because terminal rhythms such as electromechanical dissociation are common.

The relatively high mortality rate observed after our CA procedure can appear as a limitation. However, we emphasize that this rate has been calculated over the entire project (including some initial phases devoted to establish steps of our paradigm) and drastically decreased over the course of the study, reflecting the improvement of our technical expertise. Indeed, for example, in order to limit animal death we tested several epinephrine administrations routes, the retro-orbital route appearing to be the best. Thus, the residual mortality observed during the last weeks of experimentation reaching <20% is, in our opinion, acceptable and remains consistent with that expected for such experiments.

## CONCLUSION

Addressing HIE, translational research faces a challenge: there are few animal models that reproduce a pathophysiology similar to that found in the human newborn. Our aim was to develop a model approaching this as closely as possible, despite interspecies differences in cardio-vascular and cerebral tolerance to hypoxia.

We successfully developed a low-invasive protocol for an acute (9 min) hypoxic cardiorespiratory arrest in PND 14–16 rats. Since no evidence was found for memory or locomotor impairments after CA, nor evident anatomical lesions, our rat model does not represent a severe case of HIE. However, our data does point to a persistent hippocampal and likely associated behavioral impairment in CA-exposed animals, and consequently, our study should be considered as a preliminary step in the development of a new animal model for a mild form of HIE. Further in-depth investigation of the molecular, anatomical and behavioral consequences of our ischemic protocol is now required, and a refinement of the CA protocol itself should also be considered to more accurately replicate the neurobiological processes occurring in young humans.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the University of Bordeaux.

## AUTHOR CONTRIBUTIONS

OB: study conception. OB, MT-B, and JG: study design. OB and MT-B: coordination. JG, OB, LC, and MT-B: realization of the experimental procedure. JG, OB, and MT-B: data interpretation and drafting manuscript. All authors agree to be accountable for the content of this work.

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# Effects of Prenatal Exposure to Alcohol and Smoking on Fetal Heart Rate and Movement Regulation

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Negative associations of prenatal tobacco and alcohol exposure (PTE and PAE) on birth outcomes and childhood development have been well documented, but less is known about underlying mechanisms. A possible pathway for the adverse fetal outcomes associated with PTE and PAE is the alteration of fetal autonomic nervous system development. This study assessed PTE and PAE effects on measures of fetal autonomic regulation, as quantified by heart rate (HR), heart rate variability (SD-HR), movement, and HR-movement coupling in a population of fetuses at $\geq$ 34 weeks gestational age. Participants are a subset of the Safe Passage Study, a prospective cohort study that enrolled pregnant women from clinical sites in Cape Town, South Africa, and the Northern Plains region, United States. PAE was defined by six levels: no alcohol, low quit early, high quit early, low continuous, moderate continuous, and high continuous; while PTE by 4 levels: no smoking, quit early, low continuous, and moderate/high continuous. Linear regression analyses of autonomic measures were employed controlling for fetal sex, gestational age at assessment, site, maternal education, household crowding, and depression. Analyses were also stratified by sleep state (1F and 2F) and site (South Africa, *N* = 4025, Northern Plains, *N* = 2466). The final sample included 6491 maternal-fetal-dyad assessed in the third trimester [35.21 $\pm$ 1.26 (mean $\pm$ SD) weeks gestation]. PTE was associated with a decrease in mean HR in state 2F, in a dose dependent fashion, only for fetuses of mothers who continued smoking after the first trimester. In state 1F, there was a significant increase in mean HR in fetuses whose mother quit during the first trimester. This effect was driven by the Norther Plains cohort. PTE was also associated with a significant reduction in fetal movement in the most highly exposed group. In South Africa a significant increase in mean HR both for the high quit

early and the high continuous group was observed. In conclusion, this investigation addresses a critical knowledge gap regarding the relationship between PTE and PAE and fetal autonomic regulation. We believe these results can contribute to elucidating mechanisms underlying risk for adverse outcomes.

**Keywords: fetal heart rate, fetal movement, autonomic nervous system, prenatal, alcohol, smoking**

## INTRODUCTION

Many deleterious effects of alcohol consumption during pregnancy on fetal development, birth outcomes, and subsequent childhood development are well-documented (Popova et al., 2016). Prenatal alcohol exposure (PAE) is associated with increased risk of preterm birth, stillbirth, low birth weight, birth defects, and risk for neurodevelopmental disorders (Bailey and Sokol, 2011). Prenatal tobacco exposure (PTE) has also been associated with higher risk of negative outcomes including preterm birth and stillbirth as well as sudden infant death syndrome (SIDS), attention deficit hyperactivity disorder (ADHD) (Huang et al., 2018), and conduct disorder in offspring (Klonoff-Cohen and Lam-Kruglick, 2001; Cnattingius, 2004; Vardavas et al., 2010). Prior research has not elucidated a safe quantity or timing of alcohol or tobacco exposure during pregnancy, thus national guidelines in most countries advise against consuming any alcohol and smoking during pregnancy (American College of Obstetricians and Gynecologitsts, 2017; International Alliance for Responsible Drinking (Iard)., 2019).

A possible marker of effects of alcohol and smoking on fetal development is autonomic nervous system (ANS) activity as assessed through measures of fetal heart rate (HR) and heart rate variability (HRV). HRV is the variation in the heart's beat-to-beat intervals and it is regulated by the ANS, through the synergistic activity of the parasympathetic and sympathetic branches (Saul, 1990; Acharya et al., 2006). With increasing gestational age (GA), fetal HR tends to decrease while HRV increases, due to increased parasympathetic activity, maturation of central mechanisms, and more frequent occurrence of breathing movements (Cnattingius, 2004).

Studies investigating the effects of PAE on fetal HRV have obtained mixed results: some, performed during maternal intoxication, showed reduced fetal HRV (Halmesmaki and Ylikorkala, 1986; Silva et al., 1987; Schneider et al., 2008). However, two other reports found that moderate levels of acute PAE resulted in no change in fetal HR (McLeod et al., 1983; Mulder et al., 1998). In other physiological studies, McLeod and co-workers found a reduction in fetal breathing movements in response to acute alcohol exposure (McLeod et al., 1983) while Mulder et al. (1998) found no change in fetal breathing movements but observed suppressed fetal eye movements immediately following alcohol exposure. Studies investigating the effect of chronic PAE are sparse and typically retrospective. Studies reporting the effects of PTE on HR and HRV have also demonstrated mixed results. Prior studies have either reported an increase in fetal HR from acute exposure to maternal smoking (Quigley et al., 1979; Kelly et al., 1984; Péterfi et al., 2019) or no statistically significant difference in fetal HR after acute exposure to maternal smoking (Barrett et al., 1981; Goodman et al., 1984; Oncken et al., 2002). Additionally, prior studies have reported decreased HRV in response to acute maternal smoking (Eriksen et al., 1984; Goodman et al., 1984; Péterfi et al., 2019). Investigations of fetal HR and HRV in chronic maternal smokers reported decreased baseline fetal HR compared to controls and reduced HRV (Kapaya et al., 2014; Spyridou et al., 2017; Zeskind and Gingras, 2018). Chronic effects of maternal smoking have also been associated with reduced fetal breathing movements (Gennser et al., 1975), reduced fetal movement (Coppens et al., 2001), and non-reactive fetal activity-acceleration determination tests (Phelan, 1980).

In sum, research to date has primarily investigated acute effects of high levels of PAE or PTE on fetal HR or HRV. Additionally, the effects of acute and chronic maternal smoking and alcohol consumption on FHR and fetal HRV have been investigated independently of one another. This leaves a significant gap in the literature for understanding the dual effects of chronic low and moderate alcohol and tobacco use during pregnancy on function and development of fetal autonomic nervous system. The dataset analyzed in this report is a subset of the Safe Passage Study conducted by the Prenatal Alcohol and SIDS and Stillbirth (PASS) Network (Dukes et al., 2014). The aim of the study was to characterize the role of prenatal exposure to alcohol, cigarettes, and other environmental stressors on SIDS, stillbirth, and FASD. The Safe Passage Study enrolled approximately 12,000 maternal-fetal dyads in the Northern Plains (NP) of the United States (North and South Dakota) and Cape Town, South Africa (SA). Both these areas are known for high risks of SIDS, stillbirth, and FASD and have high rates of alcohol consumption and smoking during pregnancy as well as other known risk factors such as recreational drug use, or prior trauma (Bulterys, 1990; Iyasu, 2002; May et al., 2005, 2014; Popova et al., 2017). The present report focuses on reporting the effects of data driven patterns of alcohol consumption and smoking during pregnancy on measures of fetal HR, HRV movement and HR-movement coupling obtained from cardiotocographic recordings of fetuses at $\geq$ 34 weeks gestational age (GA).

## MATERIALS AND METHODS

### Participants

From 2007 until 2015, the Safe Passage Study followed the outcomes of ∼12,000 pregnancies among women from two comprehensive clinical site (CCS), one in the Cape Town area, South Africa, and one in the Northern Plains, United States.

The United States site is comprised of five clinical sites in North Dakota and South Dakota, including two sites on American Indian Reservations. In South Africa, recruitment occurred from Bishop Lavis and Belhar residential areas within Cape Town, which serve mainly the multiracial population (South African multiracial ethnic group (multiracial group), which have ancestry from more than one of the various populations inhabiting the region, including Khoisan, Bantu, European, Austronesian, and East Asian or South Asian). Screening and enrollment occurred at prenatal clinics affiliated with each CCS between 6 weeks gestation up to, but not including, delivery. Ethical approval was obtained from Stellenbosch University, Sanford Health, the Indian Health Service, and New York State Psychiatric Institute. Written informed consent to record fetal HR was part of the consent for the main study. Maternal and infant charts were abstracted to obtain demographic and relevant clinical information. We excluded participants with maternal health conditions known to affects our outcome measures (gestational diabetes, preeclampsia, hypertension), psychiatric medication use during pregnancy (SSRI's, antidepressants, classic antipsychotics, atypical antipsychotics, mood stabilizers, stimulants, antianxiety medications, or anticonvulsants), any recreational drug use during pregnancy, multiple births, and congenital anomalies.

## Self-Reported Exposure Measures

The protocol used to obtain detailed information about quantity and timing of prenatal exposure to alcohol and smoking is presented in Dukes et al. (2017). A modified Timeline Follow-back interview was employed to collect this information. Maternal smoking information was obtained through maternal interviews by trained research staff to estimate average cigarettes smoked per week for each week of pregnancy. Interviews were performed up to 4 times during pregnancy (recruitment, 20–24 weeks GA, 28–32 weeks GA, and 34–38 weeks GA). A validation study using a subset of $N$ = 108 Safe Passage Study women was performed and it indicated strong concordance between maternal report and meconium biomarkers (Himes et al., 2015). As a result of the methodology for the collection of self-report exposure information, a given participant could potentially have single or multiple segments of missing information on alcohol or smoking consumption. For this reason, we imputed missing daily exposure data using a K-Nearest Neighbor approach (Sania et al., 2020). Further information can be found in the **Supplementary Material 1**.

We then used clustering techniques to characterize multiple patterns of maternal drinking and smoking behaviors (Pini et al., 2019). Further information on alcohol and smoking exposure clustering can be found in the **Supplementary Material 2**. In the present analysis we utilized six categories of PAE (no alcohol, low quit early, high quit early, low continuous, moderate continuous, and high continuous) and a four-level PTE variable (no, quit early, low continuous, and moderate/high continuous). Depression was assessed using the Edinburgh Postnatal Depression Scale (EPDS), which has been validated for use during pregnancy, at the first study visit (Cox et al., 1987; Rubertsson et al., 2011).

## Data Acquisition and Processing

Fetal assessments were performed at 34–38 weeks gestation (Mean $\pm$ SD = 35.4 $\pm$ 1.2 weeks). Assessments were completed between 9 am and 4 pm and lasted approximately 50 min. Mothers were seated in a reclining chair or were lying supine with a 15° lateral tilt and fitted with the recording equipment. Mothers were undisturbed for the first 20 min of data collection and then answered questions on alcohol and smoking habits, recreational drug use and depression during the remaining 30 min. Fetal HR and movement data were collected using a single wide-array Doppler transducer placed on the maternal abdomen connected to a Toitu MT-320 or a MT-516 model Doppler actocardiograph (Toitu Company, Ltd., Toyko, Japan). FHR and FMOV signals were digitized at 20 Hz using a custom-built physiological data acquisition hardware and software system (DATACQ, Medelex, Inc) interfaced to a laptop computer. Specific details on the acquisition protocol can be found in previous articles (Myers et al., 2017; Shuffrey et al., 2019). Further information on data processing can be found in the **Supplementary Material 3**.

## Outcome Parameters

Mean HR and standard deviation (SD) of HR were computed for each epoch, using only the non–interpolated values. The median fetal movement was computed for each accepted fetal HR epoch except in cases where the fetal movement signal exceeded the range of the Toitu fetal movement amplifier or was not present. These cases comprised 2.5% of all records and were due to equipment failure or user error. In addition, the cross-correlation of fetal HR and movement (heart rate/movement coupling) and the lag (seconds) between movement and fetal HR derived from the cross-correlation function were computed for each accepted 4-min fetal HR epoch. The fetal HR and movement signals were first low-pass filtered between 0.002 and 0.05 Hz using a 400-point FIR filter. The fetal movement signal was z–transformed and the fetal HR was further processed by subtracting the mean from a local regression of 6 s and negative fetal HR values were set to zero (Dipietro et al., 2001). As a further control for artifact, each segment required a minimum covariance value of 0.5 and a lag at the maximum cross–correlation greater than –15 sec or less than 0 sec (i.e., changes in FMOV were required to precede changes in FHR) (Dipietro et al., 2001). For each recording, means of all the above variables were computed for the accepted segments, for each fetal state, including state 1F, also known as the quiet fetal behavioral state, and state 2F, also known as the active behavioral sleep state. More information about fetal behavioral sleep states in available in S.M.4.

## Statistical Analyses

Linear regression analyses were used to estimate the associations between exposure categories and HR and HRV and movement parameters. We fit separate models for HR mean, SD, and the cross-correlation of fetal HR and movement parameters as outcomes. All models included sex and gestational age at assessment as covariates (Dipietro et al., 2015). We additionally adjusted for maternal education (any primary school, some high school, completed high school, and beyond high school),

household crowding index (CI: 0–25th, 25–75th, and 75–100th percentile), depression scores measured with the Edinburgh scale (considered as a continuous variable), and clinical site as potential confounders. For these additional adjustments, we accounted for missing covariate data by adding a missing indicator variable in the model. Analyses were performed for all subjects combined across both sites and were repeated after stratifying by clinical site, i.e., Northern Plains and South Africa, Cape Town. Analyses were performed using R for Windows 3.6.1.

## RESULTS

In total, 11,929 mother-infant pairs were enrolled in the Safe Passage Study. We performed fetal HR assessments on 9240. Because of their known association with outcomes (Jansson et al., 2005; Rurak et al., 2011), we excluded 1098 for maternal conditions (gestational diabetes, hypertension, and preeclampsia) and congenital abnormalities and 1,236 for psychiatric medications and recreational drugs use. We also excluded 394 for incomplete exposure data and 21 for missing covariates (sex). Our final sample included a total of 6,491, 4,025 from SA and 2,466 from NP. Roughly half of the fetuses were males. **Supplementary Figure 1** shows a study consort chart.

Mean age at enrollment was 25.8 ± 5.7 years (Mean ± SD) and most participants (95.1%) had at least some high school education, and roughly half of the participants were employed. The population was composed of individuals who self-identified as white, multiracial, American Indians/Alaska natives or Other/unknown races.

A total of 51.9% of the women drank and 42.7% smoked at some point during pregnancy. Of the smokers, 14.9%, 24.1%, and 3.8% were grouped into high/moderate, low continuous and quit early groups, respectively. For alcohol 4.6%, 9.1%, 8.6%, 5.4%, and 24.3% were grouped into high continuous, moderate continuous, low continuous, and high quit early and low quit early groups, respectively.

Fetuses were assessed on average at 35.2 (± 1.3 SD) weeks of gestation. They were successively born at 39.4 (± 1.4 SD) weeks gestational age. **Table 1** contains information on maternal demographic, exposure variables, and infant characteristics, including the breakdown by study site.

**Table 2** shows the cross-tabulations of the exposure groups for the overall population and by site.

**Tables 3**, **4** show the average number of drinks and the average number of binge events per trimester and the average number of cigarettes/week per trimester. **Tables 5–12** summarize results from linear regression models discussed in the next sections.

### Significant Associations of Covariates With Fetal Physiology

In the analysis with *sites combined*, sex, GA at assessment and site were significantly associated with mean HR in 1F (Higher HR in females, $\beta = 0.85 \pm 0.29$, $p = 0.0036$; decreasing HR with increasing GA, $\beta = -0.10 \pm 0.02$, $p < 0.001$; lower HR in the Northern Plains $\beta = -2.23 \pm 0.55$, $p < 0.001$), and with mean HR in state 2F (Higher HR in females, $\beta = 1.06 \pm 0.20$, $p < 0.001$; decreasing HR with increasing GA $\beta = -0.025 \pm 0.012$, $p = 0.041$; lower HR in the Northern Plains $\beta = -2.63 \pm 0.37$, $p < 0.001$).

Sex, GA at assessment and site were all significantly related to HR-SD in both fetal states. In state 1F males had higher HR-SD than females ($\beta = -0.06 \pm 0.026$, $p = 0.023$), HR-SD decreased with age ($\beta = -0.07 \pm 0.002$, $p < 0.001$), and HR-SD was higher in fetuses from the Northern Plains ($\beta = 0.18 \pm 0.05$; $p < 0.001$). Each of these findings were also seen in state 2F ($\beta = -0.10 \pm 0.03$, $p = 0.003$; $\beta = 0.01 \pm 0.002$, $p < 0.001$; $\beta = 0.43 \pm 0.06$, $p < 0.001$, respectively).

Site was significantly associated with fetal movement in state 1F with mean levels of movement greater in the South Africa cohort ($\beta = -0.19 \pm 0.08$, $p = 0.017$).

For fetal HR/movement cross-correlation, GA at assessment and site were significant predictor (higher values with increasing GA $\beta = 0.0008 \pm 0.0002$, $p < 0.001$; Lower values in the Northern Plains $\beta = -0.017 \pm 0.0047$, $p < 0.001$), whereas for the lag of the cross-correlation sex and CI were significant (lower values for females, $\beta = -0.18 \pm 0.06$, $p = 0.0036$; $\beta = -0.86 \pm 0.41$, $p = 0.034$; $\beta = -1.00 \pm 0.40$, $p = 0.013$; $\beta = -0.94 \pm 0.41$, $p = 0.020$).

In *South Africa,* sex, GA at assessment and depression were significant predictors of mean HR in 1F (Higher HR for females, $\beta = 0.73 \pm 0.34$, $p = 0.03$; decreasing HR with increasing GA, $\beta = -0.10 \pm 0.02$, $p < 0.001$; decreasing HR with increasing depression, $\beta = -0.06 \pm 0.03$, $p = 0.025$). Sex, depression and CI 25–75th were significant predictors of mean HR in 2F (respectively, $\beta = 0.89 \pm 0.24$, $p < 0.001$; $\beta = -0.04 \pm 0.02$, $p < 0.04$; $\beta = 3.42 \pm 1.74$, $p < 0.049$). Sex was also a significant predictor of HR SD in 1F (Lower HR SD for females, $\beta = -0.06 \pm 0.03$, $p = 0.037$), while sex and GA at assessment were significant for HR SD in 2F (Lower HR SD for females, $\beta = -0.088 \pm 0.040$, $p = 0.027$, increasing HR SD with increasing GA $\beta = 0.01 \pm 0.003$, $p < 0.001$). No additional significant predictors were found for mean fetal movement. For fetal HR/movement cross-correlation, GA at assessment was significant (increasing values with increasing GA, $\beta = 0.0006 \pm 0.0002$, $p < 0.0121$), whereas for the lag of the cross-correlation sex and CI were significant (lower values for females, $\beta = -0.20 \pm 0.08$, $p = 0.01$; $\beta = -1.27 \pm 0.58$, $p = 0.027$; $\beta = -1.24 \pm 0.55$, $p = 0.023$; $\beta = -1.19 \pm 0.55$, $p = 0.030$).

In the *Northern Plains,* sex and GA at assessment were significant predictors of mean HR in 1F (respectively, $\beta = 1.37 \pm 0.58$, $p = 0.019$; $\beta = -0.10 \pm 0.03$, $p < 0.0016$), and sex and CI were significant predictors of mean HR in 2F ($\beta = 1.42 \pm 0.34$, $p < 0.001$, $\beta = 3.91 \pm 1.96$, $p = 0.047$; $\beta = 3.94 \pm 1.98$, $p = 0.047$). GA at assessment was a significant predictor of HR std in 1F ($\beta = -0.01 \pm 0.003$, $p < 0.001$), and sex, GA at assessment and education level 1 and 2 were significant predictors for HR-SD in 2F ($\beta = -0.13 \pm 0.07$, $p = 0.041$; $\beta = 0.01 \pm 0.003$, $p = 0.0021$; $\beta = 0.78 \pm 0.35$, $p = 0.025$; $\beta = 0.29 \pm 0.13$, $p = 0.023$). Sex was a significant predictor of fetal movement in 1F ($\beta = 0.16 \pm 0.07$, $p = 0.023$) and GA at assessment in 2F ($\beta = -0.009 \pm 0.003$, $p < 0.001$). Similarly, GA at assessment was significant for fetal HR/movement cross-correlations ($\beta = 0.001 \pm 0.0002$, $p < 0.001$).

In summary, expected findings of sex on autonomic regulation were observed, with females having higher HR and lower HR-SD.

**TABLE 1 |** Maternal and infant demographics and prenatal exposure information.

| | South Africa and Northers Plain (*N* = 6491) | South Africa (*N* = 4025) | Northers Plain (*N* = 2466) |
|---|---|---|---|
| **Maternal characteristics** | | | |
| Maternal age | 25.80 ± 5.72 | 24.99 ± 5.89 | 27.11 ± 5.15 |
| **Education** | | | |
| *Any primary school* | 317 (4.9%) | 289 (7.2%) | 28 (1.1%) |
| *Some high school* | 3995 (46.1%) | 2638 (65.5%) | 357 (14.5%) |
| *Complete high school* | 1324 (20.4%) | 915 (22.7%) | 409 (16.6%) |
| *Beyond high school* | 1849 (28.5%) | 177 (4.4%) | 1672 (67.8%) |
| **Married/Partnered living together** | | | |
| *No* | 2550 (39.3%) | 2087 (51.9%) | 463 (18.8%) |
| *Yes* | 3925 (60.5%) | 1923 (47.8%) | 2002 (81.2%) |
| **Employed** | | | |
| *No* | 2973 (45.8%) | 2323 (57.7%) | 650 (26.4%) |
| *Yes* | 2954 (45.5%) | 1266 (31.5%) | 1688 (68.5%) |
| Crowding index | 1.23 ± 0.89 | 1.55 ± 0.89 | 0.71 ± 0.59 |
| **Race** | | | |
| *American Indian or Alaska native* | 689 (10.6%) | 0 | 689 (27.9%) |
| *Mixed ancestry* | 4010 (61.8%) | 4010 (99.6%) | 0 |
| *White* | 1594 (24.6%) | 0 | 1594 (64.6%) |
| *Other/Unknown* | 198 (3.1%) | 15 (0.4%) | 183 (7.4%) |
| **Exposures** | | | |
| Edinburgh depression scale | 9.73 ± 6.44 | 12.57 ± 5.92 | 5.09 ± 4.18 |
| **Smoking** | | | |
| *No* | 3717 (57.3%) | 1721 (42.8%) | 1996 (80.9%) |
| *Quit early* | 244 (3.8%) | 111 (2.8%) | 133 (5.4%) |
| *Low continuous* | 1565 (24.1%) | 1335 (33.2%) | 230 (9.3%) |
| *Moderate/high continuous* | 965 (14.9%) | 858 (21.3%) | 107 (4.3%) |
| **Alcohol** | | | |
| *No alcohol* | 3120 (48.07%) | 1881 (46.73%) | 1239 (50.24%) |
| *Alcohol low quit early* | 1575 (24.26%) | 721 (17.91%) | 854 (34.63%) |
| *Alcohol high quit early* | 348 (5.36%) | 161 (4.00%) | 187 (7.58%) |
| *Alcohol low continuous* | 560 (8.63%) | 517 (12.84%) | 43 (1.74%) |
| *Alcohol moderate continuous* | 590 (9.09%) | 472 (11.73%) | 118 (4.79%) |
| *Alcohol high continuous* | 298 (4.59%) | 273 (6.79%) | 25 (1.02%) |
| **Infant characteristics** | | | |
| GA at assessment (days) | 35.21 ± 1.26 | 34.88 ± 7.05 | 35.75 ± 1.42 |
| GA at birth (weeks) | 39.39 ± 1.41 | 39.36 ± 1.47 | 39.43 ± 1.32 |
| **Sex** | | | |
| *Male* | 3221 (49.6%) | 1981 (49.2%) | 1240 (50.3%) |
| *Female* | 3270 (50.4%) | 2044 (50.8%) | 1226 (49.7%) |

GA at assessment was also significant in many associations that are consistent with the literature, with HR decreasing and variability increasing with GA (Burtchen et al., 2019). Depression was also an important covariate, with increasing values of the EDPS associated with reduced HR in SA, where many mothers presented with high scores on the EPDS questionnaire. Lastly, we observed that site was significantly associated with autonomic measures, with fetuses in SA having higher HR and lower variability compared to the NP.

## Effects of Prenatal Tobacco Exposure

In the analyses considering both *sites combined*, we found a significant association between smoking and mean HR in state 2F. A dose response effect was observed, with the low continuous group having a decrease of 0.84 ± 0.27 beats per minute (BPM, Mean ± SD) compared to non-smokers ($p$ = 0.0018) and the moderate/high continuous group having a decrease of 1.25 ± 0.32 BPM ($p$ = 0.0001) compared to non-smokers. Women who quit in the first trimester were not significantly different from non-smokers. These results are shown in **Figure 1**. We also found a significant association between smoking and mean HR in state 1F, but only for women who quit before the end of the first trimester. The HR of their fetuses was 1.91 ± 0.9 BPM per minute higher compared to the unexposed group ($p$ = 0.024).

Smoking was also significantly associated with fetal movement in both states 1F and 2F. The moderate/high continuous group

**TABLE 2 |** Cross Tabulation of smoking and drinking groups in the overall population, South Africa population, and Northern Plains population.

| | | Alcohol exposure | | | | | |
|---|---|---|---|---|---|---|---|
| | | **None** | **Low quit early** | **High quit early** | **Low continuous** | **Moderate continuous** | **High continuous** |
| **Overall population** | | | | | | | |
| Smoking exposure | *None* | 2029 | 1082 | 198 | 206 | 147 | 55 |
| | *Quit early* | 95 | 79 | 23 | 20 | 17 | 10 |
| | *Low continuous* | 638 | 277 | 76 | 226 | 248 | 100 |
| | *Moderate/high continuous* | 358 | 137 | 51 | 108 | 178 | 133 |
| **South Africa** | | | | | | | |
| Smoking exposure | *None* | 1007 | 364 | 58 | 172 | 75 | 45 |
| | *Quit early* | 41 | 35 | 7 | 19 | 8 | 1 |
| | *Low continuous* | 526 | 219 | 53 | 221 | 220 | 96 |
| | *Moderate/high continuous* | 307 | 103 | 43 | 105 | 169 | 131 |
| **Northern Plains** | | | | | | | |
| Smoking exposure | *None* | 1022 | 718 | 140 | 34 | 72 | 10 |
| | *Quit early* | 54 | 44 | 16 | 1 | 9 | 9 |
| | *Low continuous* | 112 | 58 | 23 | 5 | 28 | 4 |
| | *Moderate/high continuous* | 51 | 34 | 8 | 3 | 9 | 2 |

**TABLE 3 |** Number of drinks and binge events by trimester per alcohol group in the overall population, South Africa population, and Northern Plains population.

| | Alcohol exposure | | | | | |
|---|---|---|---|---|---|---|
| | **None** | **Low quit early** | **High quit early** | **Low continuous** | **Moderate continuous** | **High continuous** |
| **Overall population** | | | | | | |
| Total # drinks trimester 1 | 0.040 ± 0.003 | 6.014 ± 0.109 | 19.683 ± 0.379 | 2.049 ± 0.151 | 25.130 ± 0.995 | 60.671 ± 4.505 |
| Total # drinks trimester 2 | 0.018 ± 0.002 | 0.105 ± 0.130 | 0.300 ± 0.047 | 3.530 ± 0.130 | 7.501 ± 0.359 | 35.239 ± 2.921 |
| Total # drinks trimester 3 | 0.011 ± 0.001 | 0.297 ± 0.004 | 0.146 ± 0.028 | 0.872 ± 0.054 | 2.918 ± 0.163 | 17.257 ± 1.776 |
| Total # drinks in pregnancy | 0.068 ± 0.004 | 6.148 ± 0.113 | 20.089 ± 0.386 | 6.448 ± 0.212 | 35.558 ± 0.851 | 113.167 ± 5.891 |
| Total # binge events trimester 1 | 0.00 ± 0.00 | 0.43 ± 0.01 | 2.16 ± 0.03 | 0.11 ± 0.01 | 2.18 ± 0.10 | 5.71 ± 0.37 |
| Total # binge events trimester 2 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.26 ± 0.02 | 0.66 ± 0.04 | 3.79 ± 0.32 |
| Total # binge events trimester 3 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.23 ± 0.02 | 1.67 ± 0.17 |
| Total # binge events in pregnancy | 0.00 ± 0.00 | 0.43 ± 0.01 | 2.16 ± 0.03 | 0.37 ± 0.03 | 3.07 ± 0.09 | 11.17 ± 0.53 |
| **South Africa** | | | | | | |
| Total # drinks trimester 1 | 0.032 ± 0.003 | 6.056 ± 0.156 | 18.856 ± 0.533 | 1.992 ± 0.156 | 19.996 ± 1.037 | 50.951 ± 3.889 |
| Total # drinks trimester 2 | 0.024 ± 0.002 | 0.186 ± 0.026 | 0.476 ± 0.094 | 3.670 ± 0.139 | 9.081 ± 0.407 | 38.02 ± 3.118 |
| Total # drinks trimester 3 | 0.013 ± 0.002 | 0.037 ± 0.008 | 0.257 ± 0.055 | 0.825 ± 0.056 | 3.463 ± 0.190 | 18.629 ± 1.913 |
| Total # drinks in pregnancy | 0.068 ± 0.004 | 6.280 ± 0.165 | 19.589 ± 0.552 | 6.517 ± 0.222 | 32.541 ± 0.925 | 107.600 ± 5.933 |
| Total # binge events trimester 1 | 0.00 ± 0.00 | 0.47 ± 0.02 | 2.17 ± 0.04 | 0.11 ± 0.01 | 1.87 ± 0.10 | 5.21 ± 0.37 |
| Total # binge events trimester 2 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.27 ± 0.02 | 0.80 ± 0.04 | 4.11 ± 0.34 |
| Total # binge events trimester 3 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.28 ± 0.02 | 1.79 ± 0.17 |
| Total # binge events in pregnancy | 0.00 ± 0.00 | 0.47 ± 0.01 | 2.17 ± 0.03 | 0.39 ± 0.03 | 2.59 ± 0.10 | 11.12 ± 0.57 |
| **Northern Plains** | | | | | | |
| Total # drinks trimester 1 | 0.052 ± 0.005 | 5.978 ± 0.152 | 20.340 ± 0.531 | 2.734 ± 0.613 | 45.667 ± 1.756 | 166.817 ± 24.181 |
| Total # drinks trimester 2 | 0.008 ± 0.002 | 0.036 ± 0.007 | 0.743 ± 0.026 | 1.453 ± 0.330 | 1.220 ± 0.393 | 4.857 ± 3.628 |
| Total # drinks trimester 3 | 0.007 ± 0.002 | 0.023 ± 0.005 | 0.049 ± 0.020 | 1.431 ± 0.188 | 0.737 ± 0.182 | 2.273 ± 1.397 |
| Total # drinks in pregnancy | 0.068 ± 0.004 | 6.280 ± 0.165 | 19.589 ± 0.552 | 6.517 ± 0.222 | 32.541 ± 0.925 | 173.947 ± 23.854 |
| Total # binge events trimester 1 | 0.00 ± 0.00 | 0.39 ± 0.02 | 2.15 ± 0.04 | 0.07 ± 0.04 | 3.43 ± 0.20 | 11.12 ± 1.24 |
| Total # binge events trimester 2 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.07 ± 0.04 | 0.03 ± 0.02 | 0.32 ± 0.25 |
| Total # binge events trimester 3 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.28 ± 0.02 | 1.79 ± 0.17 |
| Total # binge events in pregnancy | 0.00 ± 0.00 | 0.39 ± 0.01 | 2.10 ± 0.44 | 0.14 ± 0.06 | 3.57 ± 0.20 | 11.76 ± 1.18 |

showed lower fetal movement compared to the non-smokers (1F: decrease of 0.14 ± 0.06 a.u., $p = 0.031$; 2F decrease of 0.13 ± 0.05 a.u., $p = 0.01$). These results are portrayed in **Figure 2**.

In *South Africa*, similar significant associations with smoking were observed for mean HR in state 2F, with a dose response reduction observed (Low continuous group

**TABLE 4 |** Number of cigarettes per week by trimester per smoking group in the overall population, South Africa population, and Northern Plains population.

| | Smoking exposure | | | |
|---|---|---|---|---|
| | **None** | **Quit early** | **Low continuous** | **Moderate/high continuous** |
| **Overall population** | | | | |
| Average # cigarettes/week in trimester 1 | 0.011 ± 0.0012 | 8.38 ± 0.57 | 15.57 ± 0.24 | 47.73 ± 0.70 |
| Average # cigarettes/week in trimester 2 | 0.0025 ± 0.0005 | 0.74 ± 0.13 | 15.77 ± 0.27 | 50.48 ± 0.81 |
| Average # cigarettes/week in trimester 3 | 0.0077 ± 0.0010 | 0.11 ± 0.014 | 14.75 ± 0.26 | 46.94 ± 0.80 |
| Average # cigarettes/week in pregnancy | 0.0071 ± 0.0006 | 2.86 ± 0.19 | 15.36 ± 0.22 | 48.38 ± 0.67 |
| **South Africa** | | | | |
| Average # cigarettes/week in trimester 1 | 0.008 ± 0.002 | 8.715 ± 0.574 | 16.003 ± 0.256 | 46.229 ± 0.732 |
| Average # cigarettes/week in trimester 2 | 0.002 ± 0.001 | 0.100 ± 0.232 | 17.007 ± 0.283 | 50.156 ± 0.861 |
| Average # cigarettes/week in trimester 3 | 0.006 ± 0.002 | 0.098 ± 0.021 | 15.999 ± 0.275 | 46.739 ± 0.828 |
| Average # cigarettes/week in pregnancy | 0.005 ± 0.001 | 2.971 ± 0.252 | 16.337 ± 0.229 | 47.708 ± 0.708 |
| **Northern Plains** | | | | |
| Average # cigarettes/week in trimester 1 | 0.0142 ± 0.002 | 8.111 ± 0.829 | 13.079 ± 0.678 | 59.759 ± 2.036 |
| Average # cigarettes/week in trimester 2 | 0.003 ± 0.001 | 0.053 ± 0.014 | 8.596 ± 0.678 | 53.044 ± 2.524 |
| Average # cigarettes/week in trimester 3 | 0.009 ± 0.001 | 0.115 ± 0.021 | 7.491 ± 0.566 | 48.5883 ± 2.751 |
| Average # cigarettes/week in pregnancy | 0.009 ± 0.001 | 2.760 ± 0.276 | 9.722 ± 0.511 | 53.797 ± 2.041 |

**TABLE 5 |** Linear regression results from alcohol and smoking exposure predicting Mean HR in 1F.

| **Exposure category** | **Both sites** | | | **South Africa** | | | **Northern Plains** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **N** | **Effect size Mean difference (SE)** | ***p* val** | **N** | **Effect size Mean difference (SE)** | ***p* val** | **N** | **Effect size Mean difference (SE)** | ***p* val** |
| No alcohol | 1460 | / | / | 1027 | / | / | 433 | / | / |
| Alcohol low quit early | 682 | −0.322 (0.375) | 0.390 | 413 | 0.0197 (0.4607) | 0.966 | 269 | −0.9368 (0.6607) | 0.157 |
| Alcohol high quit early | 146 | 1.135 (0.698) | 0.104 | 84 | 2.170 (0.8989) | ***0.016*** | 62 | −0.346 (1.1396) | 0.761 |
| Alcohol low continuous | 298 | −0.217 (0.518) | 0.675 | 284 | 0.0924 (0.5333) | 0.863 | 14 | −0.8672 (2.2564) | 0.701 |
| Alcohol moderate continuous | 286 | 0.202 (0.536) | 0.706 | 255 | 0.5537 (0.5722) | 0.333 | 31 | −0.8683 (1.5548) | 0.577 |
| Alcohol high continuous | 135 | 1.431 (0.742) | 0.054 | 129 | 2.0443 (0.7601) | ***0.007*** | 6 | −4.549 (3.4929) | 0.193 |
| No smoking | 1641 | / | / | 950 | / | / | 691 | / | / |
| Smoking quit early | 96 | 1.909 (0.845) | ***0.024*** | 53 | 0.9037 (1.115) | 0.418 | 43 | 3.3568 (1.3482) | ***0.013*** |
| Smoking low continuous | 766 | −0.413 (0.390) | 0.290 | 722 | −0.6005 (0.4068) | 0.140 | 44 | −0.103 (1.3928) | 0.941 |
| Smoking moderate/high continuous | 504 | −0.682 (0.450) | 0.130 | 467 | −1.0197 (0.4743) | ***0.0317*** | 37 | 1.0713 (1.4572) | 0.4624 |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

**TABLE 6 |** Linear regression results from alcohol and smoking exposure predicting Mean HR in 2F.

| **Exposure category** | **Both sites** | | | **South Africa** | | | **Northern Plains** | | |
|---|---|---|---|---|---|---|---|---|---|
| | **N** | **Effect size Mean difference (SE)** | ***p* val** | **N** | **Effect size Mean difference (SE)** | ***p* val** | **N** | **Effect size Mean difference (SE)** | ***p* value** |
| No alcohol | 2774 | / | / | 1767 | / | / | 1007 | / | / |
| Alcohol low quit early | 1362 | −0.043 (0.254) | 0.867 | 682 | 0.367 (0.342) | 0.283 | 680 | −0.5566 (0.3832) | 0.147 |
| Alcohol high quit early | 291 | 0.128 (0.467) | 0.783 | 150 | 1.1246 (0.643) | 0.0804 | 141 | −0.9834 (0.6819) | 0.149 |
| Alcohol low continuous | 530 | −0.135 (0.367) | 0.714 | 496 | 0.1759 (0.387) | 0.6498 | 34 | −0.8102 (1.32) | 0.539 |
| Alcohol moderate continuous | 553 | 0.067 (0.370) | 0.857 | 457 | 0.4089 (0.4085) | 0.3169 | 76 | −0.6608 (0.9086) | 0.467 |
| Alcohol high continuous | 275 | 0.580 (0.492) | 0.239 | 257 | 1.089 (0.518) | ***0.0356*** | 18 | −2.147 (1.803) | 0.234 |
| No smoking | 3300 | / | / | 1634 | / | / | 1666 | / | / |
| Smoking quit early | 188 | 0.395 (0.569) | 0.488 | 100 | −0.433 (0.779) | 0.578 | 88 | 1.271 (0.849) | 0.135 |
| Smoking low continuous | 1394 | −0.844 (0.271) | ***0.0018*** | 1257 | −1.0343 (0.295) | ***0.0005*** | 137 | −0.38 (0.7184) | 0.597 |
| Smoking moderate/high continuous | 883 | −1.252 (0.318) | ***0.0001*** | 818 | −1.510 (0.341) | ***0.00001*** | 65 | −0.0829 (0.9792) | 0.933 |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

**TABLE 7 |** Linear regression results from alcohol and smoking exposure predicting HR-SD in 1F.

| Exposure category | Both sites | | | South Africa | | | Northern Plains | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val |
| No alcohol | 1460 | / | / | 1027 | / | / | 433 | / | / |
| Alcohol low quit early | 682 | 0.0203 (0.034) | 0.550 | 413 | 0.0413 (0.0421) | 0.327 | 269 | −0.0256 (0.0585) | 0.661 |
| Alcohol high quit early | 146 | 0.0827 (0.0632) | 0.191 | 84 | 0.1158 (0.0822) | 0.159 | 62 | 0.0233 (0.1008) | 0.818 |
| Alcohol low continuous | 298 | 0.0711 (0.0469) | 0.130 | 284 | 0.0903 (0.0488) | 0.0641 | 14 | 0.0125 (0.1997) | 0.950 |
| Alcohol moderate continuous | 286 | 0.0973 (0.0486) | ***0.045*** | 255 | 0.1336 (0.0523) | ***0.0107*** | 31 | −0.084 (0.1376) | 0.542 |
| Alcohol high continuous | 135 | −0.0706 (0.0672) | 0.294 | 129 | −0.0392 (0.0695) | 0.5725 | 6 | −0.2214 (0.3091) | 0.474 |
| No smoking | 1641 | / | / | 950 | / | / | 691 | / | / |
| Smoking quit early | 96 | 0.0022 (0.0765) | 0.977 | 53 | −0.0655 (0.102) | 0.521 | 43 | 0.0865 (0.1193) | 0.469 |
| Smoking low continuous | 766 | 0.0197 (0.0353) | 0.577 | 722 | 0.0004 (0.0372) | 0.991 | 44 | 0.0653 (0.1232) | 0.596 |
| Smoking moderate/high continuous | 504 | −0.0236 (0.0407) | 0.563 | 467 | −0.0673 (0.0434) | 0.121 | 37 | 0.2735 (0.1289) | ***0.034*** |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

**TABLE 8 |** Linear regression results from alcohol and smoking exposure predicting HR−SD in 2F.

| Exposure category | Both sites | | | South Africa | | | Northern Plains | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val |
| No alcohol | 2774 | / | / | 1767 | / | / | 1007 | / | / |
| Alcohol low quit early | 1362 | 0.00545 (0.0442) | 0.902 | 682 | −0.0001 (0.0555) | 0.998 | 680 | 0.0407 (0.0740) | 0.582 |
| Alcohol high quit early | 291 | 0.06202 (0.08102) | 0.444 | 150 | −0.0939 (0.1045) | 0.369 | 141 | 0.2575 (0.1317) | 0.051 |
| Alcohol low continuous | 530 | 0.00782 (0.0636) | 0.902 | 496 | −0.0324 (0.0629) | 0.607 | 34 | 0.4756 (0.255) | 0.062 |
| Alcohol moderate continuous | 553 | −0.0146 (0.0642) | 0.820 | 457 | −0.0486 (0.0664) | 0.464 | 76 | 0.1333 (0.1755) | 0.448 |
| Alcohol high continuous | 275 | 0.0195 (0.0855) | 0.820 | 257 | 0.0024 (0.0842) | 0.977 | 18 | 0.0236 (0.3483) | 0.946 |
| No smoking | 3300 | / | / | 1634 | / | / | 1666 | / | / |
| Smoking quit early | 188 | −0.00164 (0.0988) | 0.987 | 100 | −0.0544 (0.126) | 0.667 | 88 | 0.0171 (0.1641) | 0.917 |
| Smoking low continuous | 1394 | 0.0216 (0.0470) | 0.646 | 1257 | 0.0147 (0.0479) | 0.759 | 137 | 0.1492 (0.1388) | 0.283 |
| Smoking moderate/high continuous | 883 | −0.0868 (0.0553) | 0.116 | 818 | −0.04703 (0.0555) | 0.397 | 65 | −0.3987 (0.1892) | ***0.035*** |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

**TABLE 9 |** Linear regression results from alcohol and smoking exposure predicting mean fetal movement in 1F.

| Exposure category | Both sites | | | South Africa | | | Northern Plains | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val |
| No alcohol | 1460 | / | / | 1027 | / | / | 433 | / | / |
| Alcohol low quit early | 682 | −0.0384 (0.0524) | 0.464 | 413 | 0.0116 (0.0673) | 0.864 | 269 | −0.1247 (0.0833) | 0.135 |
| Alcohol high quit early | 146 | 0.1518 (0.0981) | 0.122 | 84 | 0.135 (0.1319) | 0.306 | 62 | 0.239 (0.1912) | 0.212 |
| Alcohol low continuous | 298 | 0.0442 (0.0722) | 0.541 | 284 | 0.0756 (0.0780) | 0.332 | 14 | −0.3511 (0.2723) | 0.198 |
| Alcohol moderate continuous | 286 | 0.0230 (0.0748) | 0.758 | 255 | 0.0291 (0.0836) | 0.728 | 31 | 0.0899 (0.2796) | 0.748 |
| Alcohol high continuous | 135 | 0.0847 (0.1036) | 0.413 | 129 | 0.0865 (0.1113) | 0.437 | 6 | 0.6305 (0.7516) | 0.402 |
| No smoking | 1641 | / | / | 950 | / | / | 691 | / | / |
| Smoking quit early | 96 | 0.0822 (0.1175) | 0.485 | 53 | 0.131 (0.1627) | 0.421 | 43 | 0.003 (0.163) | 0.985 |
| Smoking low continuous | 766 | −0.0501 (0.0544) | 0.358 | 722 | −0.0391 (0.0594) | 0.511 | 44 | −0.0847 (0.1726) | 0.624 |
| Smoking moderate/high continuous | 504 | −0.1358 (0.0627) | ***0.031*** | 467 | −0.1085 (0.0694) | 0.118 | 37 | −0.3421 (0.1761) | 0.052 |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

decrease of 1.03 ± 0.30 BPM, $p < 0.001$; Moderate/high continuous group decrease of 1.51 ± 0.34 BPM, $p < 0.001$). A significant association with smoking was also observed in state 1F, with a decrease of 1.02 ± 0.47 BPM in mean HR for the moderate/high continuous group ($p = 0.032$).

**TABLE 10 |** Linear regression results from alcohol and smoking exposure predicting mean fetal movement in 2F.

| Exposure category | Both sites | | | South Africa | | | Northern Plains | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val |
| No alcohol | 2774 | / | / | 1767 | / | / | 1007 | / | / |
| Alcohol low quit early | 1362 | −0.0861 (0.0392) | ***0.028*** | 682 | −0.0176 (0.0527) | 0.738 | 680 | −0.172 (0.06) | ***0.004*** |
| Alcohol high quit early | 291 | 0.0486 (0.0723) | 0.502 | 150 | 0.1008 (0.0993) | 0.310 | 141 | −0.049 (0.1329) | 0.713 |
| Alcohol low continuous | 530 | −0.0641 (0.0562) | 0.254 | 496 | −0.0341 (0.0597) | 0.568 | 34 | −0.1731 (0.2017) | 0.391 |
| Alcohol moderate continuous | 553 | 0.0318 (0.0566) | 0.574 | 457 | 0.0596 (0.063) | 0.344 | 76 | −0.0904 (0.1901) | 0.635 |
| Alcohol high continuous | 275 | 0.0112 (0.0754) | 0.137 | 257 | 0.1433 (0.0799) | 0.073 | 18 | −0.1994 (0.4537) | 0.66 |
| No smoking | 3300 | / | / | 1634 | / | / | 1666 | / | / |
| Smoking quit early | 188 | 0.1095 (0.087) | 0.208 | 100 | 0.2179 (0.1199) | 0.069 | 88 | −0.0455 (0.128) | 0.722 |
| Smoking low continuous | 1394 | −0.0258 (0.0415) | 0.535 | 1257 | −0.0172 (0.0455) | 0.705 | 137 | −0.0624 (0.1102) | 0.571 |
| Smoking moderate/high continuous | 883 | −0.1258 (0.0487) | ***0.01*** | 818 | −0.1047 (0.0526) | ***0.047*** | 65 | −0.2535 (0.1476) | 0.086 |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

**TABLE 11 |** Linear regression results from alcohol and smoking exposure predicting fetal movement/HR cross-correlation Lag.

| Exposure category | Both sites | | | South Africa | | | Northern Plains | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val |
| No alcohol | 2812 | / | / | 1709 | / | / | 1103 | / | / |
| Alcohol low quit early | 1473 | −0.0399 (0.0793) | 0.615 | 677 | −0.129 (0.114) | 0.257 | 760 | 0.0549 (0.1078) | 0.611 |
| Alcohol high quit early | 310 | 0.0081 (0.144) | 0.955 | 147 | −0.331 (0.215) | 0.124 | 163 | 0.3180 (0.1884) | 0.092 |
| Alcohol low continuous | 506 | −0.2655 (0.1188) | ***0.025*** | 472 | −0.288 (0.131) | ***0.028*** | 34 | −0.5247 (0.3903) | 0.179 |
| Alcohol moderate continuous | 538 | 0.142 (0.116) | 0.222 | 428 | 0.122 (0.139) | 0.378 | 110 | 0.038 (0.226) | 0.867 |
| Alcohol high continuous | 270 | −0.0959 (0.1575) | 0.543 | 248 | −0.146 (0.174) | 0.400 | 22 | −0.2326 (0.4862) | 0.632 |
| No smoking | 3372 | / | / | 1587 | / | / | 1785 | / | / |
| Smoking quit early | 222 | 0.1859 (0.1675) | 0.267 | 105 | 0.0722 (0.252) | 0.774 | 117 | 0.3031 (0.2203) | 0.169 |
| Smoking low continuous | 1406 | −0.0445 (0.0848) | 0.600 | 1210 | −0.0015 (0.0989) | 0.987 | 196 | −0.1401 (0.181) | 0.439 |
| Smoking moderate/high continuous | 873 | 0.0497 (0.1005) | 0.621 | 779 | 0.129 (0.115) | 0.260 | 94 | −0.3993 (0.2439) | 0.102 |

*SE: standard error; P val: p-values; N = number of participants in the group. Bold and italicized values represent p-value ≤ 0.05.*

**TABLE 12 |** Linear regression results from alcohol and smoking exposure predicting HR/fetal movement cross-correlation.

| Exposure category | Both sites | | | South Africa | | | Northern Plains | | |
|---|---|---|---|---|---|---|---|---|---|
| | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val | N | Effect size Mean difference (SE) | *p* val |
| No alcohol | 2812 | / | / | 1709 | / | / | 1103 | / | / |
| Alcohol low quit early | 1473 | 0.0031 (0.0035) | 0.375 | 677 | 0.0035 (0.0048) | 0.471 | 760 | 0.0028 (0.0052) | 0.590 |
| Alcohol high quit early | 310 | −0.0022 (0.0064) | 0.727 | 147 | −0.0171 (0.0091) | 0.061 | 163 | 0.0109 (0.0091) | 0.229 |
| Alcohol low continuous | 506 | 0.0081 (0.0053) | 0.127 | 472 | 0.0064 (0.0056) | 0.255 | 34 | 0.0098 (0.0188) | 0.604 |
| Alcohol moderate continuous | 538 | 0.000061 (0.0052) | 0.991 | 428 | −0.0042 (0.0059) | 0.481 | 110 | 0.0122 (0.0109) | 0.261 |
| Alcohol high continuous | 270 | −0.0032 (0.00701) | 0.647 | 248 | −0.0028 (0.0074) | 0.704 | 22 | −0.0269 (0.0234) | 0.251 |
| No smoking | 3372 | / | / | 1587 | / | / | 1785 | / | / |
| Smoking quit early | 222 | −0.0109 (0.0075) | 0.142 | 105 | −0.0148 (0.0107) | 0.166 | 117 | −0.0049 (0.0106) | 0.646 |
| Smoking low continuous | 1406 | −0.00205 (0.00377) | 0.587 | 1210 | 0.00095 (0.0042) | 0.821 | 196 | −0.0132 (0.0087 | 0.132 |
| Smoking moderate/high continuous | 873 | 0.00203 (0.00447) | 0.650 | 779 | 0.0026 (0.0049) | 0.593 | 94 | 0.0071 (0.0118) | 0.547 |

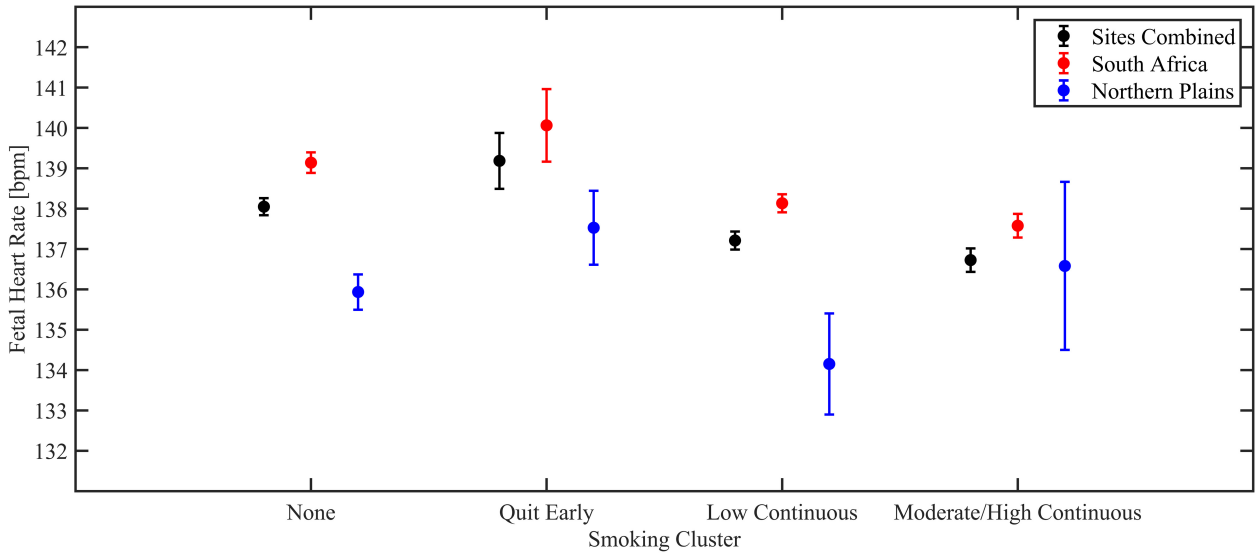


**FIGURE 1 |** Estimated marginal means from linear regression models of mean fetal HR in 2F, shown for the overall population (black), South Africa (red), and Northern Plains (blue).

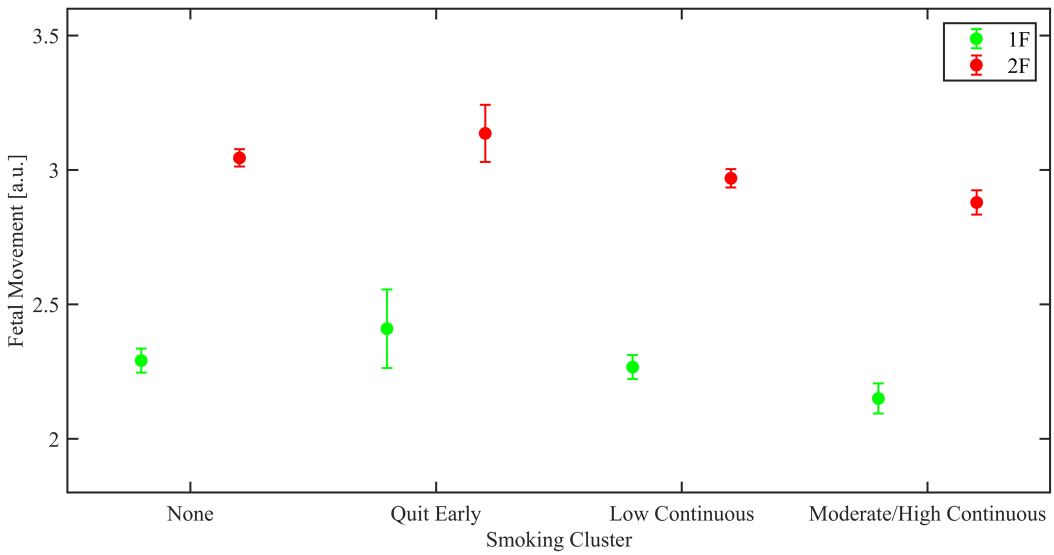


**FIGURE 2 |** Estimated marginal means from linear regression models of mean fetal movement, shown for the overall population by fetal behavioral sleep state (green 1F and red 2F).

Regarding the association of smoking and fetal movement, in South Africa subjects there was as significant reduction in fetal movement in the moderate/high group only in 2F was observed (decrease of 0.10 ± 0.05 a.u., $p = 0.047$).

In the *Northern Plains*, there was a significant increase in mean HR of 3.36 ± 1.35 BPM in 1F in fetuses of women who quit smoking in the first trimester ($p = 0.013$). In addition, there was a significant increase in HR-SD in 1F in the smoking moderate/high continuous group compared to the non-smokers (increase of 0.27 ± 0.13 BPM, $p = 0.034$), while a significant decrease in HR-SD was observed in 2F for smoking moderate/high continuous group (decrease of 0.40 ± 0.19 BPM, $p = 0.035$). In site-combined analyses similar trends to the combined were observed. Specifically, there was a reduction of fetal movement for the moderate/high group in 1F, but it did not reach significance (decrease of 0.34 ± 0.18 a.u., $p = 0.052$).

## Effects of Prenatal Alcohol Exposure

In the dataset with both sites combined, we found a significant association between PAE and the HR-SD in 1F, with an increase for the moderate continuous group by 0.10 ± 0.05 BPM ($p = 0.045$). Additionally, we found a significant reduction in

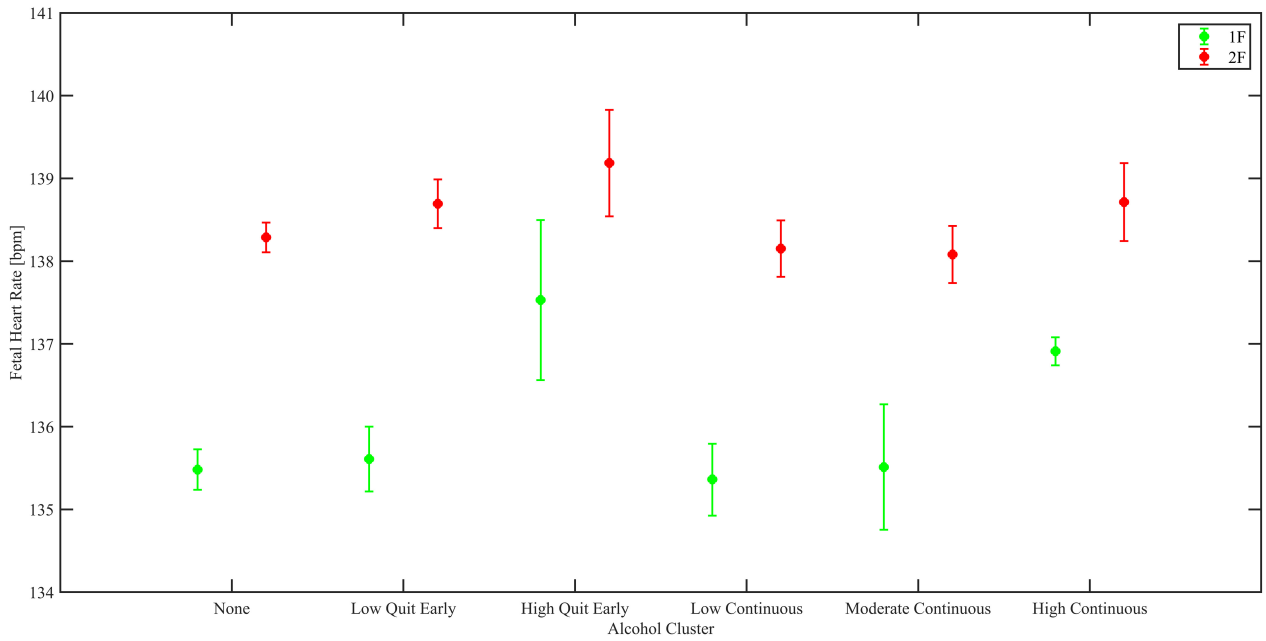


**FIGURE 3 |** Estimated marginal means from linear regression models of mean fetal HR, shown for the SA population by fetal behavioral sleep state (green 1F and red 2F).

fetal movement in 2F for the low quit early group (decrease of $0.09 \pm 0.04$ a.u., $p = 0.028$) compared to the non-drinkers.

The fetal movement/HR cross-correlation lag of the low continuous group was 0.27 s shorter than the non-drinkers ($\beta = -0.27 \pm 0.12$, $p = 0.025$).

In the *South Africa* dataset, we found an increase in mean HR in 1F both for the high quit early and the high continuous group (respectively, increase of $2.17 \pm 0.90$ BPM, $p = 0.016$; $2.04 \pm 0.76$ BPM, $p = 0.007$). Similarly, in 2F the mean HR of the high continuous group was elevated compared to the non-drinkers (increase of $1.09 \pm 0.52$ BPM, $p = 0.036$). Results are shown in **Figure 3**. The moderate continuous alcohol group had a higher HR-SD in 1F (increase of $0.13 \pm 0.05$ BPM, $p = 0.011$) compared to non-drinkers.

The low continuous group showed shorter cross-correlation lag times between movement and change in HR than the non-drinkers ($\beta = -0.29 \pm 0.13$, $p = 0.028$).

In the *Northern Plains*, we only observed a significant a decrease in fetal movement in 2F in the low quit early group compared to non-drinkers (decrease of $0.17 \pm 0.06$ a.u., $p = 0.004$).

## DISCUSSION

Several studies have reported associations of smoking and drinking during pregnancy with negative gestational outcomes and health in offspring (Schoendorf and Kiely, 1992; Scragg et al., 1993; Cnattingius, 2004; Mamluk et al., 2017; Shuffrey et al., 2020). Although these reports clearly demonstrate there are adverse effects of PAE and PTE, they do not provide information about the contributions of timing and amount of exposure on fetal ANS function. This current study addresses that shortcoming by focusing on assessments of fetal HR, HR-SD, and movement during weeks 34 to 38 of pregnancy. To our knowledge, the Safe Passage Study data set is unique in size, details of exposures, and breadth of subject characteristics.

### Prenatal Tobacco Exposure

In this study, PTE was associated with a decrease in mean HR in fetal state 2F, both in the overall population and in the sub-analysis on the SA population. These effects appeared to be dependent on dose, in that the mean decreases in HR were greatest in the fetuses of mothers who smoked at the highest levels and were not significant in fetuses of women who quit smoking during the first trimester. There were no significant PTE exposure effects in state 2F in the Northern Plans cohort; however, the number of subjects in the highest exposure group in the NP was only 65 as compared to 818 in SA. In state 1F, overall, there a significant *increase* in fetal HR in fetuses whose mothers smoked but quit during the first trimester. Broken down by site, this effect was significant only in the NP cohort. A decrease in HR in state 2F with high exposure but only in SA, and an increase in HR in state 1F in subjects whose mothers quit smoking early in pregnancy, but only in the NP suggests there are site specific factors that interact with PTE exposure, though our analyses did not reveal what these might be.

Combining both sites there were no significant effects of PTE on HR-SD. However, in the NP, the moderate-high continuous groups showed an increase in HR-SD in 1F and a decrease in 2F. The fact that PTE was associated with divergent effects in the two fetal sleep states and only in the NP site was unexpected.

However, this effect might represent less differentiated state dependent autonomic activity in some populations. In both sleep states in the combined data set PTE was also associated with a significant reduction in fetal movement in the most highly exposed group. This association was significant or approaching significance at both sites.

Tobacco cigarette smoke contains several substances which can potentially be harmful to the fetus, of these, nicotine is the most studied. Nicotine enters in the mother's bloodstream quickly and easily crosses the placenta into the fetal bloodstream (Slotkin, 1998; Cohen et al., 2005). High levels of nicotine on the fetal side of the placenta can result in a variety of adverse effects on the developing fetus (Duncan et al., 2009). Prenatal exposure to nicotine may induce functional alterations in neuronal differentiation including changes in hippocampal, cerebellar, and sensory cortex development (Shuffrey and Fifer, 2020). Previous studies looking at the acute effects of smoking on fetal autonomic regulation reported mixed findings (Lehtovirta et al., 1983; Goodman et al., 1984; Kelly et al., 1984; Ates et al., 2004; Cowperthwaite et al., 2007). Part of the reasons for the disparate findings can be attributed to the small sample size and the inconsistent characterization of patterns of smoking. Additionally, some studies investigated the acute effect of smoking while very few addressed the effect of chronic exposure. When acute effects of smoking were investigated most studies found an increase in fetal HR paired with a decrease in fetal HRV and reactivity (Goodman et al., 1984; Oncken et al., 2002), suggesting that smoking causes increased sympathetic activity.

Results from our study address the chronic effect of smoking exposure during pregnancy, which seems to go in the opposite direction of most acute studies, with a decrease in mean HR usually interpreted as a result of parasympathetic activation or sympathetic inhibition (or a combination of the two). There are few studies assessing fetuses chronically exposed to cigarette smoke. In one small study ($n$ = 13 exposed, 13 controls), exposed fetuses were observed to spend more time in a low fetal HR variation pattern and fetal activity was decreased (Coppens et al., 2001), the latter finding in agreement with our own. Kapaya et al., also found results similar to those presented in this article, with a significantly lower HR baseline in fetuses of smokers (Kapaya et al., 2014). Analogously, Duncan et al. found that fetal baboons with chronic exposure to nicotine showed an increased parasympathetic control of the heart with an increase in high-frequency HRV. These changes in HRV were associated with abnormal 5-HT-nicotine alterations in the raphe' obscurus and increased nicotinic receptor binding in the raphe' obscurus and vagal complex in the nicotine-exposed animals (Duncan et al., 2009). Prenatal exposure to maternal smoking may also result in reduced fetal oxygenation. Pathology evaluations of the placentas of smokers have shown structural changes, including a reduction in the fraction of capillary volume and increased thickness of the villous membrane when compared with non-smokers (Burton et al., 1989; Jauniaux and Burton, 1992; Larsen et al., 2002). Both factors may contribute to abnormal gas exchange within the placenta and could explain the reduced fetal movement observed in our findings (Bocking, 2003). Furthermore, it is well-known that smoking can affect fetal growth, increasing the risk for fetal growth restriction (Reeves and Bernstein, 2008). In fetuses affected by fetal growth restriction a reduction in fetal movements has been observe, potentially to conserve energy (Baschat et al., 2001). A decline in fetal movements may lead to fewer accelerations, which could induce the observed reduction in the mean HR. It is noteworthy that mean changes in fetal HR and other variables associated with smoking were small and not of immediate clinically significance in and of themselves. However, these current results support the view that chronic prenatal smoking exposure shifts the cardiac autonomic regulation to favor inhibitory actions on cardiac function.

Importantly, from a public health perspective, HR parameters of fetuses whose mothers quit smoking by the end of first trimester were not significantly different from those of non-smokers. This is in line with epidemiological findings showing that risk of stillbirth to mothers who stopped smoking during the first trimester was comparable to the risk among women who were non-smokers during the entire pregnancy (Wisborg et al., 2001) and similarly mothers who quit smoking in the first trimester have a reduced risk of preterm delivery compared to those who continued to smoke (Mainous and Hueston, 1994). Additionally, quitting before 12 weeks GA was found to diminish differences in fetal growth in comparison to non-smokers (Vardavas et al., 2010). Thus, it appears that smoking is more harmful to the developing fetus during the latter part of gestation and these findings reinforce the importance of smoking cessation early during pregnancy.

## Prenatal Alcohol Exposure

In this study there were few significant findings regarding the associations of alcohol and fetal physiology. While differences in findings between sites for the high continuous group could be due to different distributions of participants across exposure groups, it is worth noting that similar results were not observed in the high quit early groups, which had similar number of subjects in the two sites. One possible interpretation for the different site findings, is differential rates of alcohol metabolization, potentially related to body mass index (BMI). Mothers' diet can affect the fraction of body mass composed by adipose tissue, which is relevant since ingested alcohol distributes through the body water differently between lean and fat body mass (Reed, 1978).

To our knowledge, no previous studies have investigated the effects of chronic alcohol consumption during pregnancy on ANS function. Nonetheless, a few studies have investigated other aspects of fetal neurobehavior, such as behavioral states, and spontaneous and elicited startles. Hepper et al. showed that alcohol consumption delayed the decrease in the incidence of fetal startles observed with normal development. Regarding elicited startles, they found instead that fetuses exposed to alcohol were less likely to startle in response to sound than fetuses of non-drinkers (Hepper, 2007). Another relevant study from Haley et al. reported similar results to what we found but in 5–7 months old infants, who showed higher HR when exposed to high frequency drinking prenatally (Haley et al., 2006). Thus, these findings are convergent with results observed in the South Africa cohort were alterations in autonomic regulation were observed in fetuses exposed to high levels of alcohol, even if only in the first trimester.

PAE and PTE are risk factors for adverse fetal and neonatal outcomes such as intra uterine growth restriction, SIDS, and these same outcomes have been associated with altered ANS profiles (Pincus et al., 1993; Matturri and Lavezzi, 2011; Pini et al., 2021). Thus, the alterations of fetal physiology associated with PTE and PAE we describe could inform our understanding of the possible mechanisms linking PTE and PAE and adverse fetal and infant outcome. In addition, a large body of research has stressed the profound importance of the fetal environment in "programming" postnatal neurobehavioral and medical outcomes (Godfrey and Barker, 2001). The dominant theory suggests that fetuses adapt their physiology to cope with stressful environments and that, while effective in the short term these adaptations may predispose offspring to increased long term morbidity or mortality (Cao-Lei et al., 2017). Fetal HR and movement are measures of fetal well-being and maturation and have been found to be associated with later neurodevelopment (DiPietro et al., 2007; Voegtline et al., 2016). Thus, is it also possible that changes in these physiological systems in response to drinking and/or smoking are adaptations to these exposures and that adverse postnatal consequences reflect this adaptation. Regardless of mechanism, even small shifts in physiology move more individuals into low or high regions of the normal distribution. We speculate that these shifts to extreme values underly and/or are correlated with adverse outcomes associated with these toxic exposures."

Limitations of this study include the possible under-reporting of PTE and PAE due to the use of self-report measure and the lack of information on acute smoking in recordings from mothers in the low, moderate and high continuous group. We do not know the precise interval between the last cigarette that the mothers smoked and the fetal assessment. Nonetheless, given the typical time required to transport the participant and prepare for the study protocol, it is highly unlikely that women smoked a cigarette in the hour before fetal monitoring. Another limitation is the lack of precise information on time of the day of assessments, which could affect HR since fetuses start to show circadian autonomic regulation during the third trimester. In addition, part of the fetal data collection occurred while mothers were responding to questionnaires, which could have affected maternal and fetal HR regulation. Lastly, our data could reflect a potential selection bias, since the effect of alcohol and smoking on fetal autonomic parameters were not investigated in adverse pregnancy outcomes such as early delivery or fetal demise.

In conclusion, this investigation addresses a significant gap in the literature on the association smoking and drinking during pregnancy with fetal autonomic regulation. To our knowledge, this study is unique both due to the size of the cohort and the comprehensive characterization of patterns of PTE and PAE, summarized in data driven exposure groups, taking into account both timing and magnitude of exposure. We believe these results can contribute to identifying biomarkers and potentially understanding the mechanisms underlying risk for adverse outcomes.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Health Research Ethics Committee of Stellenbosch University, Sanford Health's Institutional Review Board, New York State Psychiatric Institute of Institutional Review Board, and Indian Health Service Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

ML, LS, NP, AS, MM, WF, HO, and AE contributed to the conception and design of the study. LS, CP, CF, JA, LB, LTB, and CG contributed to the acquisition. ML, JN, LS, AS, NP, and MN contributed to the analysis of data. All authors significantly contributed to the interpretation of the data and drafting the article.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.594605/full#supplementary-material

**Supplementary Figure 1 |** Study flowchart.

**Supplementary Material 1 |** Missing data imputation.

**Supplementary Material 2 |** Alcohol and smoking cluster analysis.

**Supplementary Material 3 |** Data processing.

**Supplementary Material 4 |** Fetal state coding.

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REVIEW
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# Neuromodulatory Support for Breathing and Cardiovascular Action During Development

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Neonatal survival requires precise control of breathing and cardiovascular action, with fatal consequences or severe injury without support. Prematurity presents multiple opportunities to disrupt cardiorespiratory regulation, leading to expressions of apnea of prematurity, periodic breathing, and inappropriate cardiovascular responses to apnea. Failed breathing control can result from altered breathing drives, typically arising from untimely development of sensory or motor coordination processes. Some drives, such as temperature, are a special concern in neonates with low body mass, enhancing susceptibility to rapid body cooling. Chemical drives, such as pH or $CO_2$ or $O_2$, may be inadequately developed; in some conditions, such as congenital central hypoventilation syndrome (CCHS), breathing responses to $CO_2$ or low $O_2$ may be reduced or absent, and coupling of cardiovascular responses to breathing changes are abolished. Sleep states exert profound influences on both chemical and temperature drives, with rapid eye movement (REM) sleep potentially modifying descending temperature influences, and state transitions significantly altering respiratory responses to chemical stimuli. In addition, neonates spend the majority of time in REM sleep, a state which induces a generalized inhibition of skeletal muscle activity that abolishes muscle tone to upper airway and thoracic wall muscles, enhancing the likelihood for obstructive sleep apnea. Although disrupted regulatory drives can often be replaced by positive (or negative) pressure ventilation, such as continuous positive airway pressure or enhanced by manipulating neurotransmitter action *via* caffeine, those approaches may exert negative consequences in the long term; the lungs of neonates, especially premature infants, are fragile, and easily injured by positive pressure. The consequences of caffeine use, acting directly on neural receptors, although seemingly innocuous in the near-term, may have long-term concerns and disrupts the integrity of sleep. The developmental breathing field needs improved means to support ventilation when one or more drives to respiration fail, and when the cardiovascular system, depending heavily on interactions with breathing, is compromised. Neuromodulatory procedures which manipulate the

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vestibular system to stabilize breathing or use tactile or proprioceptive stimuli to activate long-established reflexive mechanisms coupling limb movement with respiratory efforts can provide support for central and obstructive apnea, as well as for periodic breathing and cardiovascular action, particularly during sleep.

**Keywords: neuromodulation, periodic breathing, apnea, cardiovascular, proprioception, prematurity**

## INTRODUCTION

The rate of breathing and extent of air exchange in infants is normally controlled by sensing $CO_2$ and $O_2$, by core body temperature, and by voluntary breathing efforts, all of which are partially modulated by sensory feedback of lung and muscle stretch as well as by afferent signals from airflow. However, some respiratory drives, such as those from $CO_2$ or $O_2$ sensing, can be diminished, lost, or shifted in timing by altered development, or by genetic malformations, as in congenital central hypoventilation syndrome CCHS (1), or by neural trauma in the neonatal period (2). CCHS subjects show loss of $CO_2$ and $O_2$ sensing (3), as well as impaired temperature control on breathing. Other disease processes, such as Covid-19, can be accompanied by loss of the perception of dyspnea, inappropriate responses to $CO_2$ or low $O_2$, resulting in "tolerant tachypnea" and "happy hypoxemia" (4, 5).

Some instances of impaired breathing, particularly during sleep, result from disrupted cerebellar interactions with other regulatory structures, particularly the parabrachial pons. Stroke, especially with cerebellar injury, or other developmental injury with cerebellar involvement, e.g., Joubert's syndrome, is frequently accompanied by airway obstruction (2, 6), an outcome often a consequence of a loss of coordination, a principal cerebellar function, between reduced drives to upper airway muscles but continued diaphragmatic action, i.e., flaccid oropharyngeal muscles in the presence of negative thoracic pressures from a descending diaphragm. The loss of motor influences to upper airway muscles during the paralysis of REM sleep can thus result in obstructive sleep apnea (OSA). The parabrachial pons exerts a pivotal role in these sequences, coordinating signals from the cerebellum, vagal and thoracic afferents, integrating temperature and other input from more-rostral brain influences, while providing respiratory phase switching and state arousal actions (7). The state arousal functions are critical in recovery from some forms of apnea in developing infants.

The impact of sleep on reduced or distorted breathing drives extends to state influences on chemical (8), or temperature influences on breathing in early development, and can contribute to apnea of prematurity. Distortion in timing of respiratory drive influences, such as altered timing between central and peripheral chemoreceptor integration, can lead to periodic breathing. Some drives supporting breathing are lost during sleep, including voluntary breathing efforts, and the so-called "wakefulness stimulus to breathe" (9). Temperature influences on breathing, an important consideration in infants with low body mass and thus susceptible to low or high environmental temperatures, are a concern. Temperature drives are diminished during REM sleep in young or adult feline preparations (10, 11). Those REM-related effects apparently differ in premature human infants (12).

Premature infants are at high risk for cerebellar injury (hemorrhages, infarctions, and intermittent hypoxic exposure), which can compromise cerebellar growth and function (13, 14). Such cerebellar injury, seizures, or hypoxic damage can alter respiratory motor timing circuitry, or delay circulation, leading to a mismatch of central and peripheral chemoreception and modify timing of inspiratory signaling to respiratory muscles, all of which are heavily dependent on the integrity of deep cerebellar nuclei and control influences on those nuclei (15). These functional distortions can result in periodic breathing, a start-stop respiratory patterning leading to intermittent hypoxia exposure that is injurious to brain tissue (16). Such disturbed breathing patterns are common in premature infants, and can appear in full-term neonates. Severe inflammatory processes and white matter injury can occur as well (17). Appropriate and sustained inspiratory and expiratory timing must be restored to minimize periodic breathing, together with reclamation or replacements for breathing drives; that timing is heavily dependent on intact cerebellar and parabrachial pontine processes (7).

## HYPERPNEA WITH EXERCISE, AND A PHYLOGENETICALLY-OLD REFLEXIVE BREATHING DRIVE

The potential contributors to enhanced breathing efforts with exercise has been a target of respiratory physiologists for over a century. Increased ventilation to accommodate the additional metabolic demands is one possibility, but timing of the enhanced efforts before metabolic demands have built up argue against that interpretation. The range of potential mechanisms has been reviewed in detail (18), and that review described multiple processes that may be operating, with no single process providing a unifying, definitive mechanism.

A loss of drive from chemoreceptor sources, i.e., high $CO_2$ or low pH, or from low $O_2$, or insufficient temperature influences or loss of the "waking stimulus" during sleep can be overcome by recruiting a phylogenetically old reflex which couples limb locomotion with increased breathing-muscle activity. The coordination between limb movement and breathing can be easily observed in animals; a popular respiratory tale is that the distinguished French physiologist, DeJours, who loved horse racing, even in cold weather, observed synchrony between the visible expired air of horses with forward movement of the hoofs during running. Such coupling of ventilation to exercise has repeatedly been demonstrated (19, 20). In humans, children with

CCHS, who express a mutation in PHOX2B leading to loss of sensitivity to $CO_2$ and $O_2$ (3), as well as poor temperature control (21), provide an "Experiment of Nature" which illustrates the role of movement to enhance breathing. Left to sit passively, as in watching television or playing a video game, CCHS patients fail to breathe and will rapidly lose oxygenation until provoked to do so by their caretaker to voluntarily breathe (voluntary breathing drives remain intact in CCHS children). However, if such children actively move about, as in playing soccer, they ventilate adequately (22). The limb movement can be passive; simple back and forth movement of the foot will sustain breathing, and does so, even during sleep (20, 23).

The coupling of movement with breathing provides species-survival benefits. The reflex immediately augments breathing in response to locomotion when the organism is threatened, and time is not available to enhance $CO_2$ or other chemical processes usually used to increase ventilation. The independence from $CO_2$ stimulation is an important aspect in premature infants with apnea of prematurity, because ventilatory responses to increasing $CO_2$ may be poorly developed, secondary to diminished central sensitivity to $CO_2$.

The action of both foot and limb movements can enhance breathing efforts; reflexive coupling of breathing with limb movements developed early phylogenetically, and is present in all four limbs used for locomotion. Thus, in humans, movement of both the upper and lower limbs can participate in enhancing ventilation, an aspect of importance in intervening for hypoventilation in spinal cord injured patients, where proprioceptive signaling from lower limbs may be lost (24). Proprioceptive signals from muscles and tendons of the hand and feet travel *via* peripheral neural pathways to the spinal cord, and then travel up the cord *via* spinocerebellar pathways to the brain stem and cerebellum (25).

Although selected neurons in the brainstem have respiratory pacemaker qualities to influence breathing rate, the timing and extent of those breathing signals depend heavily on multiple inputs, including descending forebrain signals, which include temperature influences from the hypothalamus and affective signals from the amygdala (26), as well as afferent signals from the lung, vascular system, and thoracic wall (18). Timing and extent of those signals are coordinated by cerebellar and pontine processes which integrate signaling to nuclei mediating breathing in the medulla and pons (20, 25, 27–29). Nuclei within the medulla, in turn, send signals to the phrenic motor pools C3, C4, and C5 of the spinal cord that innervate the diaphragm, cervical and thoracic spinal nerves serving ancillary respiratory intercostal muscles, and to the hypoglossal nuclei supplying the genioglossal fibers, the protruder (dilating) muscles of the tongue (30). The processes which maintain regularity in breathing, as well as momentary processes in respiratory effort depend on multiple inputs from forebrain and brainstem sites, as well as peripheral thoracic and oral airway sensory input. The different inputs require synchronization between inputs, and timely integration to maintain sufficient drive to maintain breathing, cessation of efforts when appropriate, and appropriate interactions with cardiovascular action to maintain appropriate perfusion.

## FAILING MECHANISMS IN SLEEP-DISORDERED BREATHING; FAILING BREATHING "DRIVES" AND DISRUPTED TIMING

Several major issues confront breathing control in neonatal sleep; one is to prevent obstructive sleep apnea (OSA). A set of oro-pharyngeal muscles participate in OSA, with the tongue genioglossal muscles being principal dilators maintaining upper airway patency, and thus, a key target for OSA intervention (30). For neonatal breathing, maintaining airway patency is key to preventing OSA, but such maintenance is a concern with the generalized somatic muscle paralysis of REM sleep. The REM paralysis leads to flaccid upper airway musculature; however, diaphragmatic movements are maintained in REM sleep, producing high negative airway pressure, with the danger of collapsing the upper airway musculature, resulting in airway occlusion. Prevention of upper airway occlusion during REM sleep is thus dependent on maintaining upper airway muscle tone as well as supplying appropriate phasic *timing* of that tone to the muscles, i.e., dilating the airway slightly ahead of diaphragmatic descent and its resulting negative pressure.

A second concern in newborn breathing is protection against loss of temperature influences during the REM sleep reduction of descending hypothalamic influences. Respiratory rate is partially dependent on core temperature, which provides a significant drive to breathing in an infant with low body mass, making it susceptible to rapid core cooling when unclothed or placed in other low temperature conditions. These issues have been largely addressed in neonatal intensive care units with use of servo-controlled incubators as well as heated and humidified airflow to maintain normothermia; however, in less-developed or less-supportive circumstances, temperature control remains an issue.

A third concern is the alteration in impact of chemical sensing that accompanies state changes (8). In premature infants with apnea of prematurity, ventilatory responses to increasing $CO_2$ are immature, secondary to diminished central sensitivity to $CO_2$ (31–33). In some conditions, such as CCHS, chemical sensing of $CO_2$ is sufficiently reduced that affected children need to be mechanically ventilated during sleep. Non-CCHS children also undergo less extreme state-related breathing responses to $CO_2$ sensing, but the potential for impaired sensitivity with very early development remains a concern. Timing of inspiratory and expiratory efforts involves a complex interaction of integrating descending signals from rostral brain areas, such as affective regions of the amygdala and temperature influences from the hypothalamus, and afferent signals from stretch receptors of the lung. The cerebellum and parabrachial pons exert a coordination role in integration of these multiple inputs, especially through amygdala projections to the parabrachial pons, descending hypothalamic influences, and lung afferent projections.

Periodic breathing patterns result in successive exposure to intermittent hypoxia with each stopped-breath episode; in infants, the resulting hypoxia leads to desaturations to very low levels, with rapid restoration to normoxia with onset

of the next burst of respiratory efforts. Although periodic breathing is sometimes ignored in neonates, the consequences to the brain and other structures can be severe. The repeated desaturation/reoxygenation sequence is injurious to brain, visceral and pancreatic tissue in animal models, since periodic breathing patterns are essentially intermittent hypoxia exposures; exposure to repeated intermittent hypoxia even for short periods of a few hours results in severe cerebellar injury (16), and damage to medullary and peripheral cardiovascular integrative sites (34). In addition, reduced bone development occurs (35), as well as damage to structures mediating glucose control (36). Intermittent hypoxia episodes in human neonates lead to acute and chronic morbidities, including retinopathy of prematurity, impaired growth and cardiovascular regulation, bronchopulmonary dysplasia, sleep disordered breathing and neurodevelopmental disabilities (37–41).

Interventions to maintain ventilation in the neonatal period are few, and have the potential to impose injurious consequences. Continuous positive airway pressure (CPAP) procedures in infants pose risks, especially in the long term due to the fragility of the lungs in neonates (42) as well as concurrent bone distortion from facial masks on facial structures in early development (43). The consequences of using CPAP to manage periodic breathing in adults raise other concerns, although these aspects have not been fully explored in neonates. In adults, CPAP is often ineffective to stop Cheyne-Stokes breathing in heart failure patients (44, 45). In sleep-disordered breathing patients, major cardiovascular issues arise from use of servo-controlled positive pressure ventilation in those with periodic or Cheyne-Stokes breathing; positive pressure ventilation use leads to increased mortality in those with such breathing patterns [for rationale, see (46)]. Although adult conditions may not exactly parallel those encountered in infants, the absence of data during early development should not reduce concern of consequences of such interventions.

A common intervention to support neonatal breathing is to use methylxanthines, including caffeine, aminophylline, or theophylline, to enhance breathing drives, principally through neurotransmitter arousal processes. The benefits of caffeine, especially with early use reducing bronchopulmonary dysplasia, have been described (47). However, the long-term consequences of such use remain unclear; although a large study (48), found little change of sleep characteristics or obstructive sleep apnea in later childhood following treatment of caffeine or positive pressure ventilation to premature infants. However, concomitant arousal effects of caffeine enhance the risk for diminished sleep state integrity in prematurity, with unpredictable consequences to later cognitive development (49, 50). Thus, interventions to support breathing in premature infants might consider options other than caffeine or positive pressure ventilation to address issues of maintenance of respiratory muscle tone to prevent airway collapse, stimulation to respiratory drives to avoid central apnea, as well as synchronization of control systems to prevent periodic breathing.

Several types of impaired breathing must be considered in neonates and young infants. The first relates to loss of one or more critical drives that lead to hypoventilation or central apnea, a pattern typically found with loss of chemical sensing or temperature drive, a second relates to airway obstruction from loss of drive to the upper airway muscles with continued diaphragmatic efforts, a pattern commonly found in REM sleep or with cerebellar injury (2), and a third type of periodic breathing which stems from a loss of coordination or timing of central drive on both upper airway and diaphragmatic musculature. A solution to the first two of these issues would be to substitute a missing drive by enhancing the action by another, perhaps less commonly used influence. Correction of periodic breathing can be accomplished by assisting cerebellar-pontine mechanisms in coordination actions to overcome the stop-start patterns of periodic breathing, and stabilizing the regularity of breathing patterns. There are rather simple means to implement both solutions.

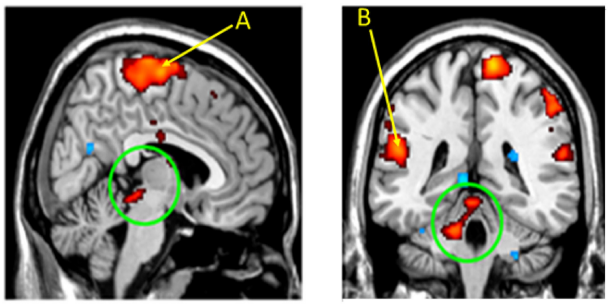


**FIGURE 1 |** Cerebellar and parabrachial pontine nuclei are activated by limb proprioceptive sensory stimulation. Functional MRI images following passive right foot movement in 14 children activated the parabrachial pons (Left circled area) and medial cerebellum (right circled area), sites involved in respiratory timing and blood pressure control. The sensorimotor cortex for the foot **(A)** and for the cervical region (for diaphragm), **(B)** are also activated, indicating that locomotion (foot) and breathing motor sites are concurrently recruited by the proprioceptive stimuli. From (51).

The evidence for coupling of breathing and limb movement processes derives from both animal physiological studies and human functional magnetic resonance studies (fMRI). We used fMRI procedures to validate in humans that lower limb proprioceptive stimulation activates cerebellar and parabrachial pontine structures (51) (**Figure 1**, left and right circled areas). In addition, the cortical area representing foot movement (**Figure 1A**) is activated, as is the cortical cervical motor area for the diaphragm (**Figure 1B**) (cervical nerves 3, 4, and 5 innervate the diaphragm).

Any intervention to support breathing must consider both the drive to upper airway muscles as well as timing of drive to those muscles. The drive aspect requires the full array of influences from airflow sensors, temperature influences, lung stretch receptors and $CO_2$ sensors for appropriate action, any of which can be poorly developed in infants. The timing aspects rely on appropriate development of cerebellar and pontine interactive circuitry, as well as chemical sensing from the periphery and central chemosensors, both of which can be distorted in timing during newborn development (52).

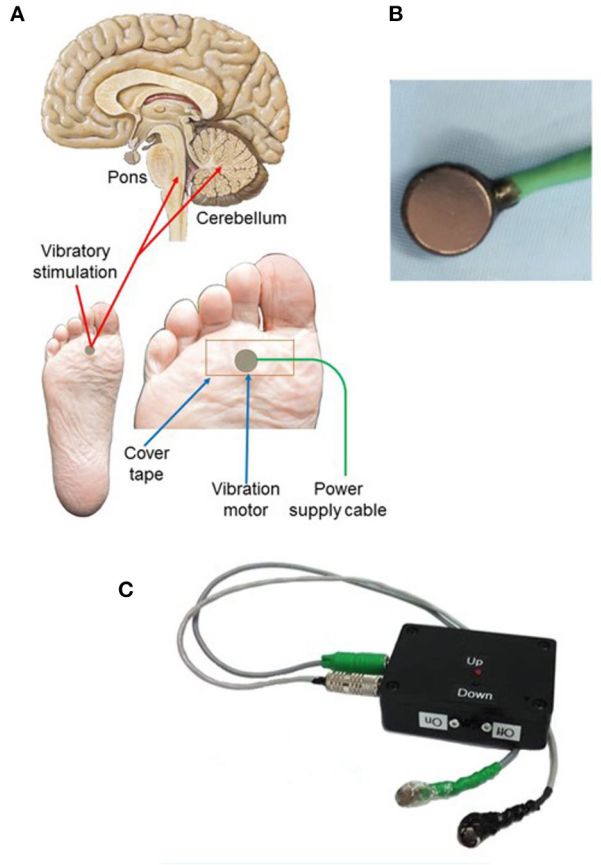


**FIGURE 2 | (A)** Placement of vibrator motor on the sole of the foot; since the limb movement/breathing reflex is operative on all four limbs (from early origins of the reflexive coupling), placement on the palm of the hand is also effective. The activated proprioceptive fibers project to the cerebellum and parabrachial pons, that in turn, integrate with motor action to the upper airway and diaphragm. **(B)** Vibrator motor with power lead. **(C)** Power supply containing two 1.5-volt fused AA batteries with two levels of vibratory stimulation. From (65).

## OVERCOMING LOST OR REDUCED RESPIRATORY DRIVES

A long history of experimental evidence indicates that a variety of "non-classical respiratory" sensory inputs can stabilize breathing patterns. Those interventions can take the form of detecting apnea, and then introducing an arousing stimulus, typically through vibration to recruit the "wakefulness stimulus" and thus enhance breathing (53). Cutaneous stimulation through rubbing of peripheral limbs has been successfully used (54), and whole-body vibratory stimulation has also been applied intermittently through vibratory mattresses (55), and lessens apnea. Vestibular stimuli, applied by oscillating a water bed (56), or manual rocking (57) show remarkable effectiveness in stabilizing breathing. There are obvious logistic issues with continuous manual rubbing intervention, incorporating whole-bed vibration, or rocking, but the interventional studies indicate that efforts to restore breathing do not necessarily have to depend on manipulating chemosensitive processes or altering arousal-related neurotransmitters through methylxanthines, or using potentially injurious positive pressure ventilation techniques.

One solution to providing another breathing drive is to use a phylogenetically old reflex that links limb locomotion with increased breathing muscle activity; running imposes increased ventilatory demands for metabolic reasons, and breathing must often increase immediately, e.g., to escape from a predator, with no time available to build up $CO_2$ signaling to breathe faster. This reflex thus bypasses the normal, but sometimes lost or reduced chemosensing and temperature drives that can occur in neonatal life or in genetic errors, such as CCHS. CCHS provides an "experiment of Nature" that allows evaluation of other means to support breathing; the condition, a consequence of PHOX2B mutation, shows an absence of $CO_2$ and $O_2$ sensitivity (3) and impaired temperature regulation, among other autonomic deficits (21). The neuromodulatory aspects of breathing support for diminished chemosensitivity were partially drawn from fMRI studies of CCHS patients who showed the reduced participation of defined cerebellar and parabrachial pontine areas to $CO_2$ challenges (58).

Early recognition of the limb movement/breathing relationship in CCHS sprung from studies relating body movement or passive limb movement to breathing (22, 59); CCHS patients who would turn blue if watching television, would ventilate normally if playing soccer. Even passive foot movement during sleep was effective in supporting breathing (23). The locomotion-breathing reflex recruits not only the diaphragm but also the upper airway and thoracic wall muscles, and can overcome the other-than-diaphragm respiratory muscle paralysis of REM sleep as well as alterations in temperature and chemical senses of that sleep state. The recruitment of upper airway muscles in addition to the diaphragm overcomes the possibility of causing obstructive apnea—unlike phrenic nerve stimulation which can result in upper airway collapse by generating a too-high negative pressure (60).

The intervention also assists timing of breathing by directly activating the cerebellum and parabrachial pons, brain sites that coordinate proprioceptive and other input to synchronize activity of the respiratory muscles. This timing coordination is critical for resolving periodic breathing, which typically results from a temporal mismatch of $CO_2$ sensing between the carotid and central chemoreceptors. CCHS patients show profound injury in cerebellar sites (21, 58, 61, 62), likely a consequence of the PHOX-2B relationships to that structure and unintended failure of ventilory support, triggering hypoxic episodes. The demonstration that foot movement can recruit enhanced breathing efforts is useful to show the principle of the phylogenetically old reflex, but that physical process of movement is obviously impractical for neonatal use. However, a variety of electrical or mechanical means can be used to simulate foot or hand movement, i.e., activate proprioceptive fibers to "trick" the brain into triggering the limb movement-breathing coupling reflex. Vibration of the foot or hand will activate proprioceptive fibers carried to the pontine and cerebellar nuclei, simultaneously recruiting action in breathing nuclei of the

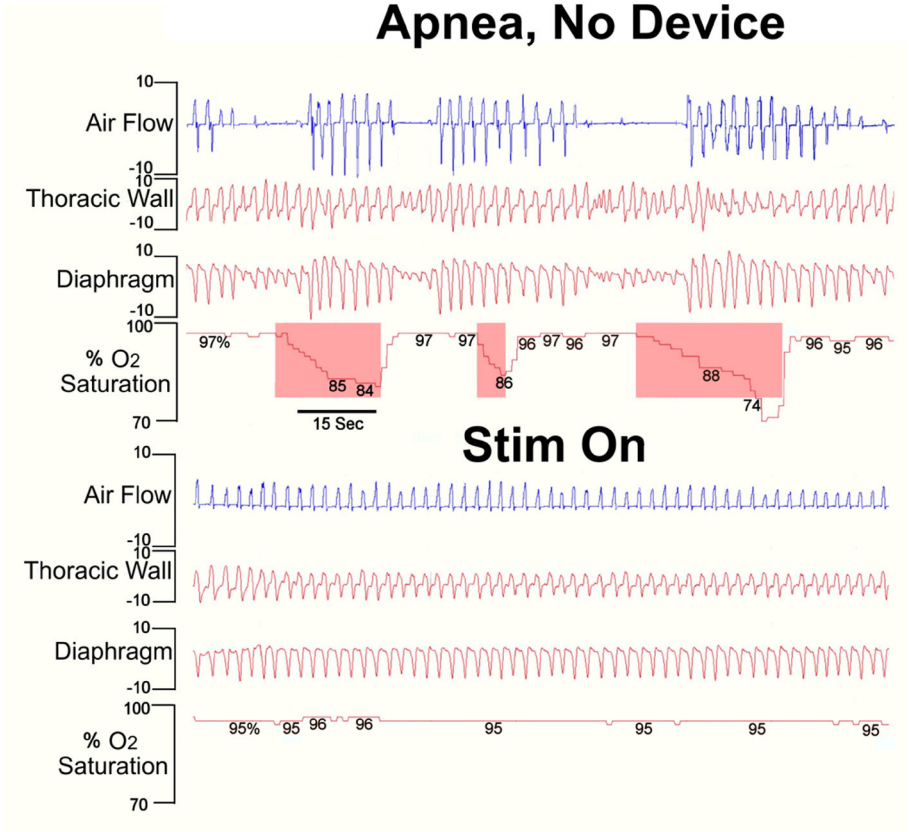


**FIGURE 3 |** Stimulation of proprioceptive fibers of the foot of a pediatric (4 years) CCHS patient without (top) and with the device (Stim On). Note the decline in $O_2$ saturation (shaded $O_2$ saturation areas following cessation of breathing efforts) during no stimulation, and the complete abolition of periodic breathing and restored saturation with stimulation. Clinical data courtesy of Dr. M. Woo [Derived from (66)].

medulla and pons, and thus activating upper airway muscles, thoracic wall muscles, and the phrenic motor pool. These processes can thus provide a "drive" to those breathing muscles, and by activating upper airway muscles, overcome the potential for obstructive sleep apnea. In addition, the diaphragmatic and thoracic wall musculature which fail in central apnea can be excited (63, 64). The neuromodulatory procedure may have particular value in other conditions, such as drug-resistant epilepsy, where a concern exists for sudden unexpected death in epilepsy (SUDEP), an outcome typically dependent on the frequency of seizures, with such seizures often accompanied by hypoxic or hypotensive periods (65).

## INTERVENTION DEVICE

Recruitment of proprioceptive fibers to activate breathing requires only simple vibration to mechanical receptors of the limbs. Such a device is shown in **Figure 2**. The objective is to apply vibration to the sole of the foot (or palm of the hand) to activate proprioceptive nerve signals, normally activated by walking or running, that rise through spinal pathways to the pons and cerebellum (**Figure 2A**). The cerebellar and parabrachial pontine sites coordinate activation of oropharyngeal muscles, including the genioglossal fibers of the tongue and muscles of the diaphragm. The vibratory device consists of small vibratory motors (**Figure 2B**) that are taped to the sole of the foot by tape. The vibratory motors, attached to the leads, are powered by two alkaline batteries, fused for safety, from a power supply box (**Figure 2C**), providing 1.5 or 3.0 volts power (switchable). The motors provide two levels of vibration amplitude at 128 Hz, a standard vibratory signal used to elicit reflexes in neurological testing. The waveform and frequency characteristics were determined after extensive empirical trials, with attention to tolerance of vibratory levels (66).

## PERIODIC BREATHING

By providing excitation to the cerebellum and parabrachial pons, timing and synchronizing effects are enhanced through proprioceptive stimulation of the limbs, and thus can reduce or abolish periodic breathing, a major concern in premature neonates, since such respiratory patterns result in serious intermittent hypoxia. The intervention can also improve cardiovascular aspects accompanying apnea, reducing bradycardia associated with the stopped-breathing periods, and assisting perfusion.

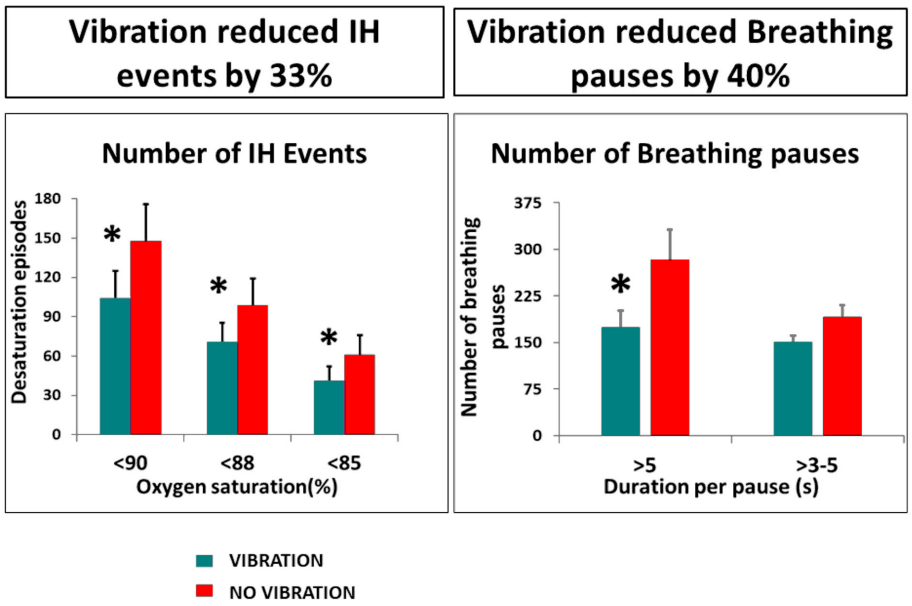


**FIGURE 4 |** Vibratory proprioceptive stimulation in 15 infants with apnea of prematurity reduced intermittent hypoxic events (IH) by 33% and breathing pauses by 40%. *$p$ <0.05. From (63).

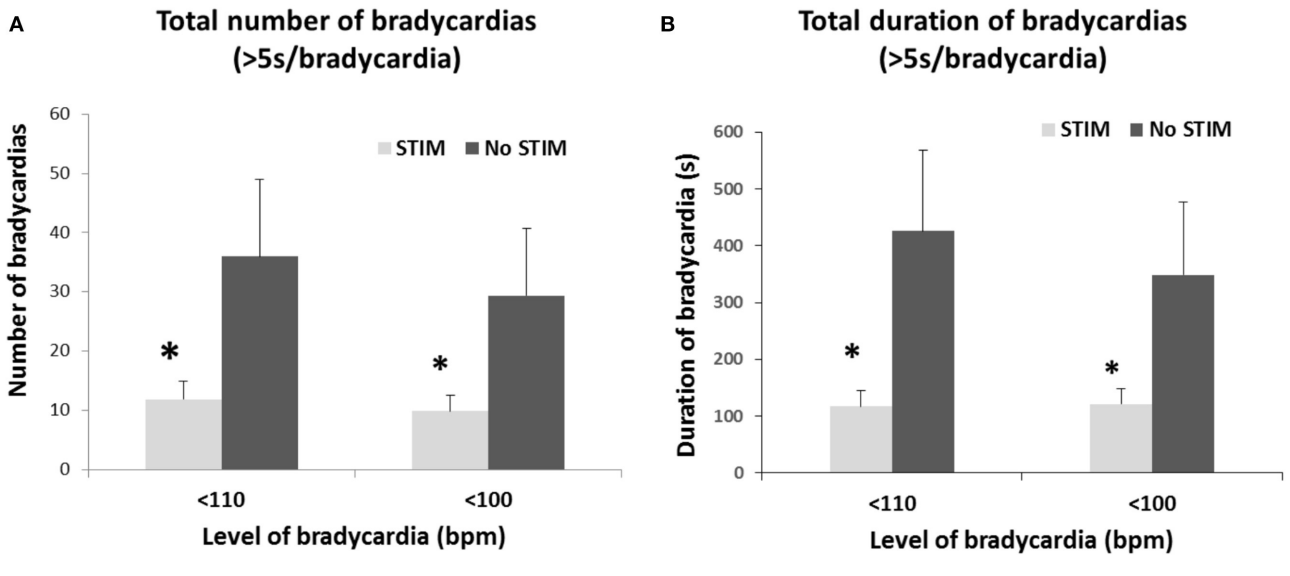


**FIGURE 5 |** Limb proprioceptive stimulation in premature infants reduced the total number **(A)** and the total duration **(B)** of mild (<110 bpm) and moderate (<100 bpm) bradycardic episodes from pre-stimulation periods. A 3-fold reduction in total duration of both mild and moderate bradycardia episodes occurred. *$p$ < 0.05. From (63).

The usefulness of the intervention in managing periodic breathing can be seen in **Figure 3**, which shows breathing in an awake CCHS child with and without the vibratory device. The CCHS patient desaturates to low 70% values, but those values remain at full saturation with stimulation.

The device applies transcutaneous vibration to the soles of the foot and palms of the hand to elicit nerve signaling from pressure and other limb proprioceptor sensors to pontine, cerebellar, and medullary brain areas that coordinate limb movement and reflexively activate brain areas controlling breathing. The procedure enhances a reflexive drive to breathing when other breathing drives fail from disease processes or during sleep. The device is of use in obstructive sleep apnea, central apnea, periodic breathing, and hypoventilation, and will also normalize extremes of change in blood pressure to respiratory events (63, 64, 66).

It is important to note that the mode of action used by recruitment of reflexive breathing drives differs from the intuitive mechanism of arousing the apneic subject to restore the "wakefulness" drive to breathe. Such an approach can be effective for restarting breathing, but repeatedly arouses the subject, making a night's sleep not restful. Instead, the principle is to recruit an ancient reflexive drive that maintains sleep integrity (24, 63).

The safety, suitability and efficacy of the procedure has been shown in a premature infant trial (63), that demonstrates a significant reduction in number and duration of long breathing pauses and intermittent hypoxic events, as well as the number and duration of bradycardic events (**Figures 4**, **5**). The intervention provides an ancillary drive to breathing which replaces missing drives during development, allowing stabilization of breathing, diminishing apnea, and reducing extreme changes in cardiovascular patterns accompanying breathing pauses.

## ALTERNATIVE OPTIONS FOR NEONATAL AND ADULT APNEA

The concerns for breathing support in neonates also extend to adults with sleep-disordered breathing. The principal intervention has been CPAP, which has serious deficiencies in patient adoption, since the devices are uncomfortable, are often noisy, are not compact, making travel difficult, has humidity control issues, and long-term support for blood pressure is not assured (67, 68), and can be dangerous for periodic breathing use (46). Mandibular positioning devices have been adopted for mild or moderate adult OSA, but are not considered for neonatal cases, and are often considered inadequate for severe adult apnea. Such devices pose a potential for temporal mandibular joint injury (from forward positioning of the mandible), and do nothing for central apnea (in conditions where patients lack central drive to breathe for all respiratory muscles, not just upper airway musculature). Genioglossal/hypoglossal nerve stimulation requires invasive surgical implantation of an electrical stimulation device, coupled with placing leads to the 12th cranial nerve or to genioglossal fibers of the tongue. The procedure is inappropriate for neonates. The surgery is expensive, is coupled with a risk for infection, damage to nerves and other tissue from stimulation leads, and may require multiple surgical interventions to restore the subcutaneous power supply of the implanted device. Mechanical positive pressure ventilation can be used, but also poses a concern of injury to delicate or compromised lungs, such as those in young or medically-compromised patients.

## CONCLUSIONS

Disordered breathing in early life can lead to impaired oxygenation, often with intermittent hypoxia exposure, which can induce severe injury to multiple brain sites. Disordered breathing can result from failure of chemical, temperature, or state-related motoric drives; particular sleep states enhance the appearance of some of these failures. Alterations in drives can further compromise breathing by delayed timing or inappropriate coordination of afferent influences, or disrupt integration of sensory input with motor outflow to upper airway and diaphragmatic muscles. Multiple interventions have been used to demonstrate how potential injury from positive pressure procedures or bypassing caffeine administration can be avoided. These interventions attempt to modulate sensory input, include tactile and vibratory bed stimulation, and often rely on recruiting arousal actions to restore breathing. Vestibular input, applied through oscillatory water beds or rocking, has been employed successfully to exert excitatory vestibular/cerebellar processes to activate and synchronize breathing with motion input. Loss of breathing drives can be overcome by enhancing ancient reflexive interactions between limb locomotion and respiration, and that enhancement can be accomplished artificially by proprioceptive activation, "tricking" the brain into perceiving that the limbs are moving. If sufficient stimulation is provided, the proprioceptive signals can enhance cerebellar and pontine processing to improve coordination of integrative processes and abolish periodic breathing.

## AUTHOR CONTRIBUTIONS

RH and KK contributed equally to the design and implementation of the research and findings outlined in this manuscript. Both authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The University of California has been issued a patent for a device described in this review, listing RH as one of the inventors.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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